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Clinical Pathology of the Serum Electrolytes

Edited by

F. W. Sunderman
and
F. W. Sunderman, Jr.

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CLINICAL PATHOLOGY
of the
SERUM ELECTROLYTES

CLINICAL PATHOLOGY *of the* SERUM ELECTROLYTES

Edited by

F. WILLIAM SUNDERMAN, M.D., Ph.D., Sc.D.
Director, Institute for Clinical Science
Director of Education, Association of Clinical Scientists
Clinical Professor of Medicine, Jefferson Medical College
Philadelphia, Pennsylvania

and

F. WILLIAM SUNDERMAN, Jr., M.D.
Director of Clinical Laboratories and
Associate Professor of Pathology
University of Florida College of Medicine
Gainesville, Florida

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Contributors

GONZALO E. APONTE, M.D.
Jefferson Medical College
Philadelphia, Pennsylvania

WALTER F. BALLINGER, II, M.D.
Johns Hopkins University School
of Medicine, Baltimore, Maryland

J. THEODORE BRIERRE, Jr., M.D.
U.S. Naval Medical School
Bethesda, Maryland

MAURICE B. BURG, M.D.
National Heart Institute, NIH
Bethesda, Maryland

ALBERT CANNON, M.D.
University of South Carolina
College of Medicine
Charleston, South Carolina

JOHN E. CARROLL, B.S.
Jarrell-Ash Corporation
Waltham, Massachusetts

SIDNEY S. CHERNICK, Ph.D.
National Institute of Arthritis
and Metabolic Diseases, NIH
Bethesda, Maryland

JAMES E. CLARK, M.D.
Jefferson Medical College
Philadelphia, Pennsylvania

THOMAS E. CONE, Jr., M.D.
Children's Hospital Medical Center
Harvard Medical School
Boston, Massachusetts

ELGIN C. COWART, M.D.
U.S. Naval Medical School
Bethesda, Maryland

ADELBERT CRAMER, M.D.
U.S. Naval Hospital
St. Albans, New York

GERALD S. DEAN, M.D.
Highland Park, Hospital
Highland Park, Illinois

HERBERT DERMAN, M.D.
City of Kingston Laboratories
Kingston, New York

LARRY G. DICKSON, M.D.
U.S. Naval Medical School
Bethesda, Maryland

BASIL DOUMAS, Ph.D.
University of Alabama
School of Medicine
Birmingham, Alabama

KURT M. DUBOWSKI, Ph.D.
University of Oklahoma
School of Medicine
Oklahoma City, Oklahoma

HARRY C. EHRMANTRAUT, Ph.D.
Mechrolab, Inc.
Palo Alto, California

JAMES B. FIELD, M.D.
University of Pittsburgh
School of Medicine
Pittsburgh, Pennsylvania

ALFRED H. FREE, Ph.D.
Ames Research Laboratory
Elkhart, Indiana

HELEN M. FREE, B.S.
Ames Research Laboratory
Elkhart, Indiana

RICHARD H. GADSDEN, Ph.D.
University of South Carolina
College of Medicine
Charleston, South Carolina

DAVID S. GERBARG, M.D.
Kingston General Hospital
Kingston, New York

JOSEPH S. HANDLER, M.D.
National Heart Institute, NIH
Bethesda, Maryland

GERALD G. HOFFMAN, M.D.
Lake Forest Hospital
Lake Forest, Illinois

G. S. HUBER, CMDR, MSC, USN
U.S. Naval Medical Research Institute
Bethesda, Maryland

J. DE LA HUERGA, M.D., Ph.D.
Grant Hospital, Northwestern
University Medical School
Chicago, Illinois

JOHN P. KALTENBACH, Ph.D.
Northwestern University Medical
School, Chicago, Illinois

BERNARD J. KATCHMAN, Ph.D.
Department of Research, Miami
Valley Hospital, Dayton, Ohio

CARROLL M. LEEVY, M.D.
New Jersey College of Medicine
Jersey City, New Jersey

HARRISON H. LEFFLER, M.D.
Professional Laboratories
Washington, D.C.

LEO LUTWAK, Ph.D., M.D.
Graduate School of Nutrition
Cornell University
Ithica, New York

ROBERT P. MacFATE, Ph.D.
Division of Laboratories
Board of Health
Chicago, Illinois

SAMUEL NATELSON, Ph.D.
Roosevelt Hospital
New York, New York

HANS N. NAUMANN, M.D.
Veterans Administration Hospital
Memphis, Tennessee

IRWIN H. ROSENBERG, M.D.
II and IV Harvard Medical Services
Boston City Hospital
Boston, Massachusetts

LEON E. ROSENBERG, M.D.
National Cancer Institute, NIH
Bethesda, Maryland

JOSEPH C. SHERRICK, M.D.
Passavant Memorial Hospital
Northwestern University School of
Medicine, Chicago, Illinois

G. MILTON SHY, M.D.
University of Pennsylvania
School of Medicine
Philadelphia, Pennsylvania

GEORGE W. SMETTERS, M.D.
Chicago Wesley Memorial Hospital
Chicago, Illinois

GEORGE F. STEVENSON, M.D.
Holy Cross Hospital
Silver Spring, Maryland

JON V. STRAUMFJORD, Jr., M.D., Ph.D.
University of Alabama
School of Medicine
Birmingham, Alabama

F. WILLIAM SUNDERMAN, M.D., Ph.D.
Jefferson Medical College
Philadelphia, Pennsylvania

F. WILLIAM SUNDERMAN, Jr., M.D.
University of Florida College of
Medicine, Gainesville, Flordia

JENO E. SZAKACS, M.D.
U.S. Naval Hospital
St. Albans, New York

HENRY A. TELOH, M.D.
Ingalls Memorial Hospital
Harvey, Illinois

DAVID M. TRAVIS, M.D.
University of Florida College of
Medicine, Gainesville, Florida

DONALD P. TSCHUDY, M.D.
National Cancer Institute, NIH
Bethesda, Maryland

HARRY F. WEISBERG, M.D.
Chicago Medical School
Chicago, Illinois

EARL B. WERT, M.D.
Mobile Infirmary, Mobile, Alabama

THOMAS E. WHEELER, Ph.D.
U.S. Naval Medical School
Bethesda, Maryland

ROBERT E. ZIPF, M.D.
Department of Research, Miami
Valley Hospital, Dayton, Ohio

Preface

This book contains the edited proceedings of an Applied Seminar on the Serum Electrolytes in Clinical Medicine, held in Washington, D.C. under the auspices of the Association of Clinical Scientists. In organization and format this volume is similar to the published proceedings of five previous seminars:

Lipids and the Steroid Hormones in Clinical Medicine

Measurements of Exocrine and Endocrine Functions of the Pancreas

Evaluation of Thyroid and Parathyroid Functions

Clinical Pathology of Hemoglobin, Its Precursors and Metabolites

Serum Protein and the Dysproteinemias

Clinical scientists, working in research and diagnostic laboratories, are vitally concerned with principles and problems of methodology as well as the clinical interpretations of the results of their analyses. The *Applied Seminars* attempt to bridge these areas of laboratory and clinical medicine and to provide comprehensive reviews on specific topics in clinical science.

The topic of the present seminar was selected in order to acquaint clinical scientists with newer procedures for measurements of serum electrolytes and with the rapid advances in our knowledge of physiologic regulations of electrolyte metabolism. A number of the analytical procedures described in this book are currently being undertaken primarily in research laboratories. In our opinion, many of these procedures, such as X-ray spectroscopy, atomic absorption spectrometry, osmometry and gas chromatography, will find increasingly important applications in future years. Likewise, the chapters pertaining to physiologic and clinical interpretations emphasize the newer concepts of neuro-hormonal control and active transport of electrolytes which have emerged from recent clinical investigations. It is our hope that clinical scientists will find the sections on methodology helpful in initiating newer procedures in their laboratories and the sections on physiology and clinical interpretations helpful in applying the newer concepts of electrolyte metabolism to their patients at the bedside.

Our appreciation is expressed to the lecturers and assisting physicians who have generously contributed their time and energies to the success of the *Applied Seminar* and to the preparation of these proceedings. Our thanks are given to our publishers, and particularly to Mr. Payne E. L. Thomas and Mr. Warren H. Green for their gracious cooperation and Mrs. Robin George for her invaluable secretarial assistance.

F. WILLIAM SUNDERMAN, M.D.
F. WILLIAM SUNDERMAN, JR., M.D.

1833 Delancey Place
Philadelphia, Pennsylvania 19103

Contents

PART I – ANALYTICAL CONSIDERATIONS

Page

Preface vii

Chapter

1. Introduction: Fundamental Considerations of Electrolytes in Biological Fluids 3
F. William Sunderman, M.D., Ph.D.

2. The Chemical Measurement of Sodium in Biological Fluids

A. General Considerations 8
Robert E. Zipf, M.D., and Bernard J. Katchman, Ph.D.

B. Gravimetric Method for the Measurement of Sodium in Serum . . . 13
F. William Sunderman, M.D., Ph.D.

C. Colorimetric Method for the Measurement of Sodium in Serum . . . 15
Robert E. Zipf, M.D., and Bernard J. Katchman, Ph.D.

3. The Chemical Measurement of Potassium in Biological Fluids

A. General Considerations of the Cobalti-nitrite Method for the Measurement of Potassium in Serum 19
Richard H. Gadsden, Ph.D., and Albert Cannon, M.D.

B. Cobalti-nitrite Method for the Measurement of Potassium in Serum . . 27
Richard H. Gadsden, Ph.D., and Albert Cannon, M.D.

C. Platinic-chloride and Tetraphenylboron Methods for the Measurement of Potassium 31
F. William Sunderman, Jr., M.D., and
F. William Sunderman, M.D., Ph.D.

4. The Chemical Measurement of Calcium in Serum 37
Robert P. MacFate, Ph.D.

A. General Considerations 37

B. Oxalate-permanganate Method for the Measurement of Calcium in Serum 48

C. N-Hydroxynaphthalimide Method for the Estimation of Calcium in Serum 51

5. The Chemical Measurement in Biological Fluids

A. General Considerations 56
F. William Sunderman, Jr., M.D., and
F. William Sunderman, M.D., Ph.D.

B. Magnesium Ammonium Phosphate Method for the Measurement of Magnesium in Serum 60
F. William Sunderman, M.D., Ph.D., and
F. William Sunderman, Jr., M.D.

Chapter Page

C. Bohuon Method for the Measurement of Magnesium in Serum . . . 62
Jon V. Straumfjord, Jr., M.D., Ph.D., and Basil Doumas, Ph.D.

6. MEASUREMENTS OF ULTRAFILTRABLE CALCIUM AND MAGNESIUM IN SERUM . . 66
F. William Sunderman, Jr., M.D.

7. THE MEASUREMENT OF CHLORIDE IN BIOLOGICAL FLUIDS 74
J. de la Huerga, M.D., Ph.D., G. W. Smetters, M.D., and Joseph C. Sherrick, M.D.

A. General Considerations 74

B. Mercuric Chloranilate Method for the Estimation of Chloride in Biological Fluids 77

C. Mercurimetric Method for the Estimation of Chloride in Serum . . 80

D. Argentimetric Method for the Measurement of Chloride in Biological Materials 81

E. Discussion of Chloride Methodology 83

8. THE MEASUREMENT OF INORGANIC SULFATE IN BIOLOGICAL FLUIDS 90
Thomas E. Wheeler, Ph.D., J. Theodore Brierre, Jr., M.D., and Larry G. Dickson, M.D.

A. General Considerations 90

B. Benzidine Method for the Measurement of Inorganic Sulfate . . . 94

9. THE MEASUREMENT OF INORGANIC PHOSPHORUS IN SERUM 97
John P. Kaltenbach, Ph.D., Gerald S. Dean, M.D., and Gerald G. Hoffman, M.D.

A. General Considerations 97

B. Molybdate Method for the Measurement of Inorganic Phosphorus in Serum 101

10. THE MEASUREMENT OF ORGANIC ACIDS IN SERUM

A. General Considerations 104
Alfred H. Free, Ph.D., and Helen M. Free, B.S.

B. Measurement of Lactic Acid in Serum and Blood 107
Alfred H. Free, Ph.D., and Helen M. Free, B.S.

C. Measurement of Ketone Bodies in Blood and Serum 109
Sidney S. Chernick, Ph.D.

D. Gas Chromatographic Method for Fractionation of Fatty Acids in Serum . 113
Alfred H. Free, Ph.D., and Helen M. Free, B.S.

11. THE MEASUREMENT OF AMMONIA IN SERUM 119
Harrison H. Leffler, M.D.

Chapter Page

12. THE MEASUREMENT OF CARBON DIOXIDE AND pCO_2 IN SERUM 124
Kurt M. Dubowski, Ph.D.

A. Gasometric Method for Carbon Dioxide in Serum 124

B. Graphical Computation of Carbon Dioxide Tension in Serum . . . 128

C. Electrometric Method for pCO_2 in Serum 138

13. THE ELECTROMETRIC METHOD FOR BLOOD pH 143
Jeno E. Szakacs, M.D., and Adalbert Cramer, M.D.

14. THE CHEMICAL MEASUREMENT OF TOTAL BASE IN SERUM

A. General Considerations 157
Hans N. Naumann, M.D.

B. THE HALD BENZIDINE METHOD FOR THE MEASUREMENT OF TOTAL BASE . 163
Hans N. Naumann, M.D.

15. THE MEASUREMENT OF SERUM CONDUCTIVITY FOR THE ESTIMATION OF TOTAL BASE AND SODIUM

A. General Considerations 166
F. William Sunderman, M.D., Ph.D.

B. ESTIMATION OF SERUM TOTAL BASE BY THE SUNDERMAN CONDUCTIVITY METHOD 172
George F. Stevenson, M.D.

16. THE MEASUREMENT OF OSMOLALITY OF SERUM

A. The Freezing Point Method for the Measurement of Osmolality . . . 176
F. William Sunderman, M.D., Ph.D.

B. The Vapor Pressure Method for the Measurement of Osmolality . . . 181
Harry C. Ehrmantraut, Ph.D.

17. FLAME PHOTOMETRIC METHOD FOR SERUM CATIONS (SODIUM, POTASSIUM, CALCIUM AND MAGNESIUM) 189
Henry A. Teloh, M.D.

18. ATOMIC ABSORPTION SPECTROMETRY OF CALCIUM AND MAGNESIUM IN BIOLOGICAL FLUIDS 199
F. William Sunderman, Jr., M.D. and John E. Carroll, B.S.

19. X-RAY SPECTROSCOPIC METHOD FOR SERUM ELECTROLYTES 209
Samuel Natelson, Ph.D.

PART II – PHYSIOLOGICAL CONSIDERATIONS

20. THE ROLE OF THE ADRENALS IN THE METABOLISM OF SODIUM AND POTASSIUM . 228
James E. Clark, M.D.

21. THE ROLE OF SODIUM IN THE TRANSPORT OF NON-ELECTROLYTES 233
Irwin H. Rosenberg, M.D., and Leon E. Rosenberg, M.D.

Chapter Page

22. THE REGULATION OF CALCIUM AND PHOSPHORUS METABOLISM 242
Leo Lutwak, Ph.D., M.D.

23. THE REGULATION OF MAGNESIUM METABOLISM 248
Donald P. Tschudy, M.D.

24. THE REGULATION OF CHLORIDE METABOLISM 253
George W. Smetters, M.D., Joseph C. Sherrick, M.D., and J. de la Huerga, M.D., Ph.D.

25. THE REGULATION OF SULFATE METABOLISM 260
Joseph T. Brierre, Jr., M.D., Thomas E. Wheeler, Ph.D., and Larry G. Dickson, M.D.

26. THE pH OF SERUM CEREBROSPINAL AND INTRACELLULAR FLUIDS IN ACUTE ACID-BASE DISTURBANCES 265
David M. Travis, M.D.

27. THE REGULATION OF WATER METABOLISM
A. The Regulation of Osmotic Pressure 273
Joseph S. Handler, M.D.

B. Abnormalities of the Secretion of Antidiuretic Hormone 276
Donald P. Tschudy, M.D.

28. PHYSIOLOGIC DISTURBANCES OF FLUID AND ELECTROLYTES IN DIARRHEA . . 280
G. S. Huber, Cmdr., MSC, USN

29. PHYSIOLOGIC REGULATION OF ELECTROLYTES IN CARDIAC FAILURE 288
Maurice B. Burg, M.D.

PART III – CLINICAL CONSIDERATIONS

30. SERUM ELECTROLYTES IN INANITION, FEVER AND HYPOTHERMIA 293
F. William Sunderman, M.D., Ph.D.

31. ALTERATIONS IN ELECTROLYTES IN RENAL DISEASE

A. Serum Electrolytes in Renal Insufficiency 299
F. William Sunderman, M.D., Ph.D.

B. Electrolyte Metabolism in Renal Disease 306
Ira N. Tublin, M.D.

C. Renal Lesions Associated with Electrolyte Imbalance 310
Gonzalo E. Aponte, M.D.

32. DISTURBANCES OF FLUID AND ELECTROLYTE METABOLISM IN INTESTINAL OBSTRUCTION 332
Walter F. Ballinger, II, M.D.

33. ALTERATIONS OF ELECTROLYTE METABOLISM DURING GENERAL ANESTHESIA . . 337
Harry Weisberg, M.D.

Chapter Page

34. The Serum Electrolytes in Pneumonia and Other Infectious Diseases . 349
Herbert Derman, M.D., and David S. Gerbarg, M.D.

35. Fluid and Electrolyte Metabolism in Liver Disease 353
Carrol M. Leevy, M.D.

36. Electrolyte Metabolism in Diabetes Mellitus 362
James B. Field, M.D.

37. Disturbances of Electrolyte Metabolism in Bone Diseases and Parathyroid Diseases 368
Leo Lutwak, Ph.D., M.D.

38. Electrolytes in the Pathogenesis of the Periodic Paralysis 373
G. Milton Shy, M.D.

39. Disturbances of Electrolyte Metabolism in Selected Pediatric Problems 388
Thomas E. Cone, Jr., M.D.

40. Alterations of Serum Electrolytes in Burns 394
Jeno E. Szakacs, M.D., and Elgin C. Cowart, M.D.

41. Alterations of Serum Electrolytes in Intoxication with Methanol, Bromides and Salicylates 402
Earl B. Wert, M.D.

Index 407

CLINICAL PATHOLOGY
of the
SERUM ELECTROLYTES

Introduction

Fundamental Considerations of Electrolytes in Biological Fluids

F. WILLIAM SUNDERMAN, M.D., Ph.D.

The electrolyte or mineral metabolism in health and disease has been intensively studied by many investigators during the past hundred years. It is sometimes a little frustrating to find that new approaches to current topics of medical importance are merely extensions of studies undertaken scores of years ago. In 1840, Berzelius[1], the Swedish chemist, studied the electrolytes in muscle and reported the ash to contain sodium chloride and lactate, potassium chloride, magnesium, and calcium phosphate. A few years later in 1847, Liebig[2] noted that muscle ash was rich in potassium but poor in sodium, while blood serum, from which the muscle was nourished, was rich in sodium and contained relatively little potassium. These same problems are being studied today, although the approaches and interpretations of the data may vary.

Among all animals, no matter of what order, indubitable biochemical similarities are found. Water, for example, is the predominating component in their bodies, and their mineral constituents always include the bicarbonates, chlorides, sulfates, and phosphates of sodium, potassium, calcium, and magnesium. The current concept of the partition of body fluids in man has been developed from the studies of Gamble[3]. The body fluids in man constitute about 70 per cent of the body weight, and it is convenient to divide them into two major fractions: (a) intracellular fluids, which constitute 45 to 50 per cent of the body weight and (b) extracellular fluids, about 25 per cent of the body weight. The extracellular fluid may be subdivided into two parts: (a) the vascular fluid representing about 5 per cent, and (b) the interstitial fluid, comprising about 20 per cent of the body weight. The vascular and interstitial fluids together constitute the extracellular fluid. Under normal conditions, the volumes of the body fluid compartments are relatively constant.

In recent years, the physicochemical state of the water in biocolloidal solutions has aroused much interest. Many of the older theories suggested that the water in biologic materials may exist in several different forms, such as occlusion water, absorption water, capillary water, and chemically bound water. By the term "free" water, it was understood that such water is capable of having solutes dissolved in it, in contradistinction to "bound" water, which is incapable of dissolving solutes. The hypothesis has been developed that biologic materials contain both "free" and "bound" water. The pros and cons of this hypothesis will not be discussed except to state that within the limits of error in the measurements (about ±2 per cent), it would appear that all the water in the body is in the "free" state[4].

It may be desirable for the sake of clarity to mention here the terminology used in studies of water and electrolyte metabolism. Since the concentrations of molecules and ions in dilute solutions are of more importance than the mere weights of the salts in solutions, it is convenient to use as a measure of concentration a unit that is related to the number of molecules and ions present. The units that express such relations are spoken of as moles and millimoles, equivalents and milliequivalents. The convenience of this notation is evident, if it is remembered that one milliequivalent of an acid, when it combines with one milliequivalent of a base, forms one milliequivalent of a salt.

Although the body fluids of all animals are composed of salt solutions having similar components, nevertheless, the *concentrations* of the salts may be quite different, depending not only upon the type of animal but upon the type of body fluid. Of the various fluids of the body, the one that is most acces-

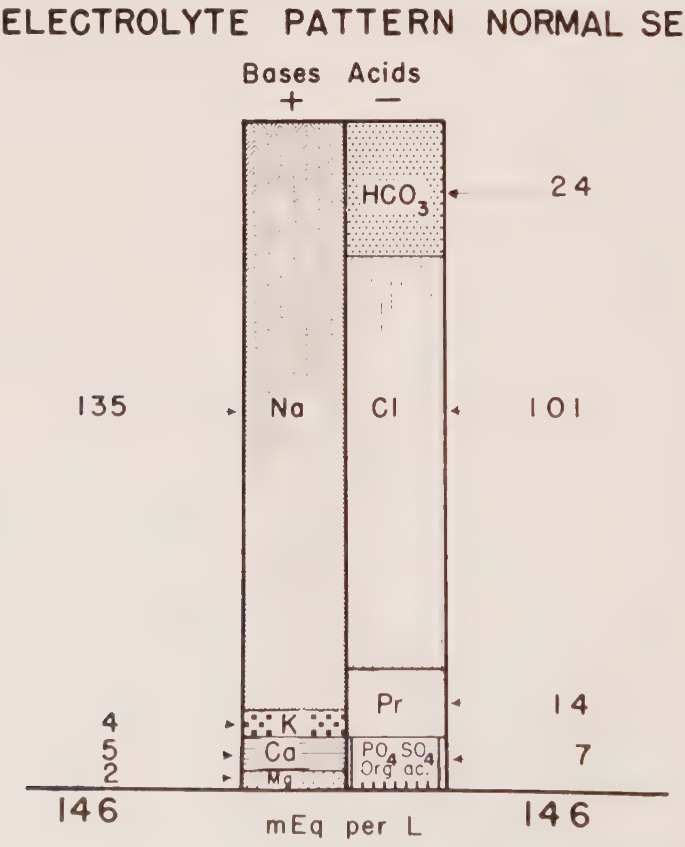

Fig. 1

sible for chemical analysis during life is the blood serum. This is one of the main reasons that emphasis is placed on the serum pattern in studies of electrolyte equilibria. The normal electrolyte pattern of serum is shown on Figure 1. It will be seen that the bases or cations are Na, K, Ca, and Mg; the acids or anions are HCO_3, Cl, protein, organic acids, PO_4 and SO_4. Since the pH of serum is on the alkaline side of the isoelectric point of the serum proteins, the proteins combine with base and behave as anions. At the sides of Figure 1 are given average normal values for the concentrations of the serum components. Under normal conditions, the mean concentration of the total electrolytes (total base) is approximately 146 mEq. per L. This concentration is maintained constant within a narrow range of about ±3 mEq.

The electrolyte composition of the extracellular fluid has been more accurately determined than that of the intracellular fluid because of the availability of serum and transudates for direct chemical analyses. The composition of the intracellular fluid is almost certainly not uniform among the various tissues and cannot be measured directly by chemical analyses. The accepted values are either derived from radioisotope dilution studies *in vivo* or from estimates based upon desiccation studies of tissues *in vitro*.

Although the concentrations of electrolytes in the various body fluids may differ strikingly from each other, there is strong evidence to suggest that the concentrations are adjusted so that they are all isotonic with each other; or in other words, they have the same osmotic pressure.

It is recognized that small variations in the total osmotic pressure of the extracellular fluids impair the efficiency of cells and that large changes may destroy them. Our cells have developed an unusual requirement for an environmental medium of constant concentration and composition. Abrupt changes in the medium create functional disturbances in the cells. If these changes occur in moderate degree, the organism becomes ill; if the changes are severe, entire groups of cells become functionless and, as a consequence, death ensues.

Under normal conditions, the total osmotic pressure of the body fluids is constant within a remarkably small range of variation. The prevention of dislocations in the osmotic pressure is principally an activity of the kidney. In renal failure, however, extensive changes may occur in the metabolic processes and cause alterations in the chemical composition of the body fluids. As a result of these alterations, changes occur in their total osmotic pressure.

It would seem appropriate to comment briefly upon the significance of the total osmotic pressure. It is recognized that the composition of the serum and extracellular fluids may differ strikingly from the composition of the cells and intracellular fluids. Despite this fact, there appears to be little doubt that these fluids are in osmotic equilibrium, or are isotonic with each other. The serum is in osmotic equilibrium with the interstitial fluid, and the interstitial fluid similarly is in osmotic equilibrium with the intracellular. It has been shown that the sum of the osmotically active electrolytes in the corpuscles equals the sum of the osmotically active electrolytes in the serum, when the calculations are made on a water basis, even though the concentrations of the individual ions in the corpuscles and serum are not equal. Since membranes of the cells of the body allow free passage of water, it would seem obvious that the total osmotic pressure should be the same for all media of the body.

The osmotic pressure is a force inherent in all solutions and is determined by the concentration of dissolved components of all kinds. In dilute solutions of nonelectrolytes, such as urea and glucose, the osmotic pressure depends upon the total number of nonionized molecules that are present per unit of solvent. In dilute solutions of electrolytes, such as sodium chloride and sodium bicarbonate, the osmotic pressure will depend upon the number of dissociated ions of sodium, chloride, and bicarbonate in addition to the number of molecules of sodium chloride and sodium bicarbonate that are undissociated.

When solutes are contained in a solvent to form a solution, four properties may be studied in relation to the solvent: (a) vapor-pressure lowering, (b) boiling-point elevation, (c) freezing-point lowering, and (d) osmotic pressure. These are sometimes called colligative properties of solutions, since the measurement of any one of them may be converted into that of any of the others. For clinical purposes, osmotic pressure is usually expressed in terms of freezing-point depression to which it is proportional.

One mole of a solute dissolved in a kg. of water lowers the freezing-point 1.858 C. The depression of the freezing-point in normal sera averages 0.56 C. Therefore, the total osmotic pressure of serum expressed in osmolar concentration is $\frac{0.56}{1.858}$ or 302 milliosmols. Of these 302 milliosmols contributing to the total osmotic pressure, approximately 290 (or 96 per cent) are electrolytes; the remaining 12 milliosmols are non-electrolytes. Normally, glucose and urea together constitute about 10 of these 12 milliosmols. Although normally the nonelectrolytes exert only about 4 per cent of the total osmotic pressure, nevertheless, in pathologic conditions, this percentage may be appreciably increased.

When the total osmotic pressure becomes either decreased or increased to any marked degree, the protoplasmic structure of the organism will not withstand the strain and death occurs. In our measurements, limits beyond which protoplasmic changes are irreversible correspond to freezing-point depressions below 0.49 and above 0.65° (i.e., 265 to 350 milliosmols). In our experience, no patient has recovered when the serum osmotic pressure was outside of this range.

An additional mechanism that also helps to regulate the interchange of water and electrolytes in the body compartments is diagrammatically portrayed in Figure 2 in accordance with the Starling hypothesis.[5, 6] The lengths of the arrows have no significance in this diagram. There is a constant interchange of water, electrolytes, and non-electrolytes between the blood stream and the tissue spaces through the capillary membranes. The capillary blood pressure tends to drive the fluid out of the capillaries, whereas the serum proteins, which are unable to escape through the capillary walls, tend to draw the fluids back into the capillaries. On the other hand, the interstitial fluid proteins, which are normally of low concentra-

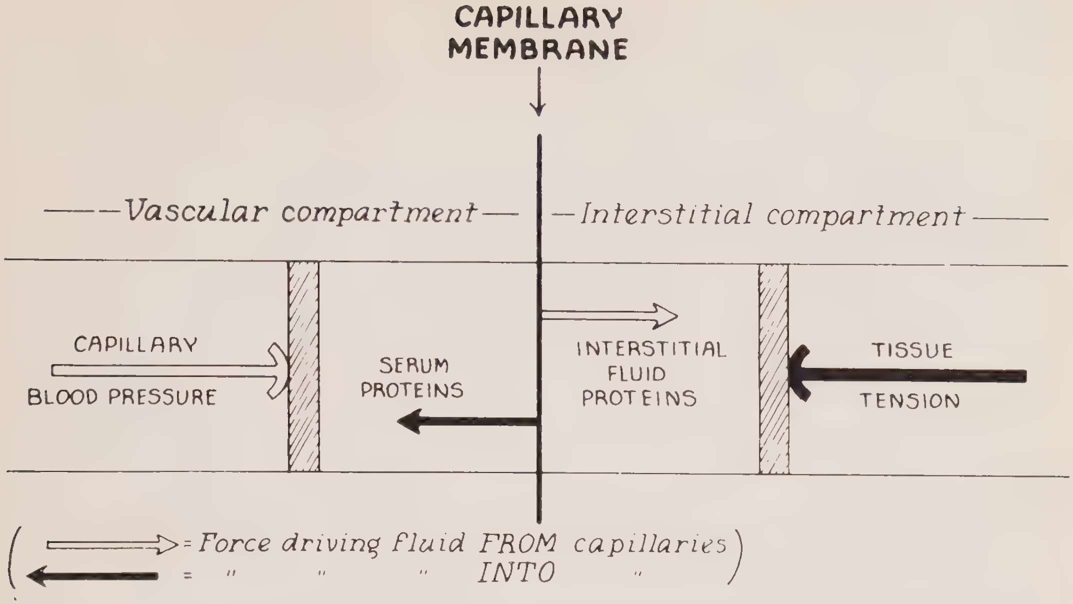

Fig. 2. Colloidal osmotic pressure and hydrostatic pressure in the interchange of fluids across the capillary membrane.

tion, tend to draw the fluids from the capillaries. The pressure exerted by the elastic subcutaneus tissues and designated as "tissue tension" is a factor of importance in driving fluid back into the capillaries. The forces driving fluid from the capillaries are the capillary blood pressure and the interstitial fluid proteins; the forces driving fluid into the capillaries are the serum proteins and tissue tension.

In summary, any consideration of electrolyte metabolism must take into account three inherent tendencies of the organism that contribute to the movement of body fluids.

1. Constancy of the volume of various body fluids.
2. Constancy of the concentration of solutes in body fluids.
3. Maintenance of osmotic equilibrium in body fluids.

All of these factors are normally maintained constant within a narrow range.

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The Chemical Determination of Sodium in Serum

A. General Considerations

ROBERT E. ZIPF, M.D. and BERNARD J. KATCHMAN, Ph.D.

INTRODUCTION

The need for quantitative methods for the analysis of sodium in biological materials is obvious. The classical macro-gravimetric methods are the most exact. However, they are time-consuming, requiring large samples, and being most suitable for the analysis of urine and feces. For the analysis of serum, it is necessary to have either at least a 5 ml. sample or a micro balance. The macro techniques are therefore of limited applicability.

The advent of the gravimetric zinc uranyl acetate method of Kolthoff for sodium permits small sample analysis with the technique of macro-gravimetry. The uranyl method is as accurate as the classical methods due to the great weight of the precipitate which is formed with sodium. When potassium is much less than sodium in the sample, the uranyl method is even more exact than the classical gravimetric methods. Gravimetric methods, in general, are not used in clinical laboratories since they are too time-consuming.

Sodium has been determined in biologic fluids by a wide variety of physical and chemical techniques. These are summarized in Table I.

GRAVIMETRIC METHODS

Macro Method

The classical macro-gravimetric procedures require wet-ashing (Kjeldahl type digestion) or dry-ashing (electric furnace at 400° C)[1] as a preparatory step. This is followed by precipitation of phosphate and sulfate with $BaCl_2$ in alkaline medium. Subsequently, barium, calcium, and magnesium are precipitated with ammonium carbonate. The filtrate is evaporated to dryness. At this point, it is necessary to remove some of the unprecipitated barium and calcium by additional chemical manipulations. Eventually, the sodium and potassium are isolated as sulfates; the combined sulfates are ignited at 600 to 700° C., and the cooled residues are weighed. From the combined sulfates, potassium is precipitated as the chloroplatinate by chloroplatinic acid. After washing with alcohol to remove excess chloroplatinic acid and washing to remove sodium sulfate, the precipitate is dried and weighed. From the weight of the combined Na_2SO_4 and K_2SO_4 and the weight of K_2PtCl_6, the sodium weight is calculated by difference.

MICRO METHOD

The commonly-used micro-gravimetric procedure is based upon the precipitation of sodium as the complex triple salt, uranyl zinc sodium acetate[2, 3]. According to Peters and Van Slyke[1], phosphate needs to be removed, as it will interfere as precipitated uranyl phosphate. However, there is some question as to whether or not phosphorus also interferes in the analysis of serum. The method in current use is Sunderman's modification[4] in which phosphate is not removed. This simplifies the procedure.

TABLE I DETERMINATION OF SODIUM IN BIOLOGIC FLUIDS

PRECIPITATE	QUANTITATION	REFERENCE
Chemical Methods		
Sodium and Potassium Sulfates	Gravimetry	1
Uranyl Zinc Sodium Acetate	Gravimetry	2-4
Sodium Pyroantimonate	Gravimetry	5
Sodium Pyroantimonate	Titrimetry	6, 7
Ion Exchange Resins	Titrimetry	8
Sodium αmethoxy Phenyl Acetate	Titrimetry	9
Uranyl Zinc Sodium Acetate	Titrimetry	10, 11
Uranyl Zinc Sodium Acetate	Titrimetry	12
Uranyl Zinc Sodium Acetate	Titrimetry	13
Uranyl Manganous Sodium Acetate	Photometry	14
Uranyl Magnesium Sodium Acetate	Photometry	15
Sodium Violurate	Photometry	16
Uranyl Zinc Sodium Acetate	Photometry	17, 18
Uranyl Zinc Sodium Acetate	Photometry	19
Uranyl Zinc Sodium Acetate	Photometry (salicylate)	20
Uranyl Zinc Sodium Acetate	Photometry (peroxide)	21, 22
Uranyl Zinc Sodium Acetate	Photometry (ferrocyanide)	23, 24
Physical Methods		
Neutron Activation Analysis		25, 26
Flame Photometry		
Electrometric Determination – Special Electrode		27-29
Conductivity		30

Serum (1 ml.) is digested with sulfuric acid- ferric sulfate mixture and then ashed at 550° to 600° C. The ash is resuspended in distilled water, the suspension is clarified by centrifugation; and an aliquot of the clear solution is evaporated to dryness. Uranyl zinc acetate reagent is added to the dried residue, and the precipitate is collected on a weighed Gooch filter crucible. After several washings with reagent, alcohol and ether, the precipitate and crucible are dried in a vacuum desiccator over calcium chloride and weighed.

The gravimetric pyroantimonate method proposed by Kramer and Tisdall[5] has had only limited application in the analysis of sodium in biological materials due to difficulties encountered by most investigators. The crystalline precipitate has the formula $Na_2H_2Sb_2O_7 \cdot nH_2O$. Apparently, the inconstant water of crystallization and extensive coprecipitation made gravimetry difficult. The titrimetric method developed to overcome these difficulties will be discussed.

TITRIMETRIC METHOD

The titrimetric procedures for sodium which have been reported are based upon iodometric, oxidimetric, alkalimetric, and complexometric titration procedures.

Rourke[7] modified and simplified the procedure of Kramer and Gittleman for the iodometric analysis of sodium pyroantimonate, $Na_2H_2Sb_6O_7 \cdot nH_2O$. According to Rourke's method, sodium may be precipitated quantitatively directly from an alcoholic solution of serum or plasma when the alcohol is added at 10°C., so that the proteins do not precipitate. The antimony of the precipitate is determined by iodometric titration. The pentavalent Sb is reduced by KI to the trivalent state, and the iodine liberated is titrated with thiosulfate.

$$Sb^{+5} + 3I^- \rightarrow Sb^{+3} + I_3^-$$
$$I_3^- + 2S_2O_3^= \rightarrow 3I^- + S_4O_6^=$$

This simple technique permitted the analysis of sodium with as little as 0.3 grams of plasma or serum.

Oesper and Pauerstein[9] precipitated sodium as the salt of α-methoxyphenyl acetic acid, which forms a compound of the formula $C_6H_5CH\ (OCH_3)\ COOH \bullet C_6H_5CH(OCH_3)\ COONa$ which is sparingly soluble in organic solvents but dissolves readily in hot water. The precipitate can be washed, redissolved, and titrated with standard alkali. Samples analyzed in the range of 103 to 175 mEq. per L. of sodium showed deviation of 3 per cent from that obtained with a flame photometer.

Clark *et al.*[11] developed a simple, alkalimetric procedure based upon a titration of the acetate in the complex uranyl zinc sodium acetate. Sodium is precipitated from deproteinized serum as the complex triple salt. The precipitate is dissolved in CO_2- free distilled water and titrated with standard alkali.

$$2(UO_2)_3ZnNa(CH_3COO)_9 + 18NaOH \rightarrow$$
$$3Na_2U_2O_9 + 2Zn(CH_3COO)_2 + 14Na^+CH_3COO^- + 9H_2O$$

In this titration each sodium atom is equivalent to nine atoms of sodium hydroxide, a favorable factor. With this method, twenty samples of human serum of between 0.60 and 0.90 gram were analyzed, and these gave an average value for sodium of 130.7 ± 0.2 mEq. per L. Duplicates do not differ by more than 1 per cent.

Vanatta *et al.*[8] developed a unique procedure for the isolation of serum sodium which permits acidmetric titration. They report that total analysis can be accomplished in two hours on 1.0 ml. serum with an average error of less than 2 per cent, compared to the gravimetric method of Butler and Tuthill[1, 3] Sodium is removed from serum by means of a cationic resin, Amberlite IR-112. The sodium is then removed from the resin column with $BaC1_2$. Barium is precipitated with H_2SO_4, and the Na^+C1^- is converted to Na^+OH^- by passage through a strong anionic exchange resin, Amberlite IR-400 in the hydroxide form. The sodium as NaOH is titrated with standard acid.

A complexometric method[13] has been developed which is based upon the fact that the zinc in the uranyl zinc sodium acetate triple salt can be complexed with, and therefore titrated with, ethylenediamine tetraacetate; dithizone is used as the indicator for the detection of end-point.

The titrimetric method (oxidimetry) for uranium has been applied to the analysis of sodium, inasmuch as the ratio of sodium to uranium in the uranyl zinc sodium acetate precipitate is a constant[31]. Ball[12] adapted this method to the analysis of serum sodium. Ashed serum samples (0.2 ml.), when analyzed by this method, agree within 1 per cent with the gravimetric procedure of Butler and Tuthill[3]. Sodium is precipitated from the ashed serum sample as uranyl zinc sodium acetate. The precipitate is dissolved in acid, and the sexivalent uranium is reduced in a Jones reductor to the quadrivalent state.

$$UO_2^{++} + Zn \xrightarrow[\text{Jones Reductor}]{} UO^{++} + ZnO$$

Quadrivalent uranium is oxidized with standard dichromate with ferric iron as a catalyst and a suitable indicator (sodium diphenylamine sulfonate).

$$\begin{array}{l} 3UO^{++} + 6Fe^{+++} + 3H_2O \rightarrow 3UO_2^{++} + 6Fe^{++} + 6H^{+} \\ 6Fe^{++} + Cr_2O_7^{=} + 14H^{+} \rightarrow 6Fe^{+++} + 2Cr^{+++} + 7H_2O \\ \hline 3UO^{++} + Cr_2O_7^{=} + 8H^{+} \rightarrow 3UO_2^{++} + 2Cr^{+++} + 4H_2O \quad \text{Overall Reaction} \end{array}$$

PHOTOMETRIC METHODS

The remainder of the methods for sodium analysis which will be discussed require colorimetric or photometric analysis of a colored reaction product.

Two variations on the usual uranyl zinc sodium acetate precipitate have been reported. Maruna[15] precipitated sodium from a protein-free filtrate as uranyl magnesium sodium acetate. This precipitate was dissolved in alkali and read at 420 mμ. This method obeys Beer's Law between 8 and 80 mg. per 100 ml.

Leva's[14] micromethod requires only 0.04 ml. of blood. In this procedure, sodium is precipitated as uranyl manganous sodium acetate, and the manganous ion is oxidized to permanganate by KIO_4. The permanganate color is read in a colorimeter.

A number of photometric methods are based upon precipitation of sodium as uranyl zinc sodium acetate. The simplest technic, from the standpoint of laboratory manipulation, is that developed by Bradbury[19]. Bradbury[19] simply read the supernatant or residual reagent at 425 mμ. after centrifugation of the precipitated triple salt. This technic checked with the gravimetric method within 2 per cent.

The precipitate of uranyl zinc sodium acetate, when redissolved in water, has an absorption spectrum with a peak about 430 mμ. Hoffman[17] showed that by stabilization of the color with NH_4SCN, serum sodium could be analyzed with an accuracy of 1 per cent or less. The method of Albanese and Lein[18], which omits the NH_4SCN, can achieve an accuracy ± 3 per cent.

Many methods have been developed to enhance the color of the triple salt. For example, uranyl salts form peroxides which have the $(UO_4) \cdot 3H_2O$ formula[32]. In alkaline solution, these uranyl peroxides form colored complexes[21, 22]. Stone *et al.*[21] reported a rapid method for serum and urine. Sodium is precipitated as uranyl zinc sodium acetate, and precipitate is dissolved in $(NH_4)_2CO_3$ and H_2O_2. In the range of 130 to 350 mg per 100 ml., the color is read at 460. mμ.

McCance and Shipp[23], and later Salit[24], used $K_4Fe(Cn)_6$ to form colored complexes with zinc and UO_2^{++} in acetic acid solutions of the triple salt.

Butterworth[20] dissolved the triple salt in water and obtained an orange colored solution upon the addition of 1 per cent sodium salicylate.

Muraca and Bonsack[16] developed a procedure for serum sodium based upon the fact that alkali and alkaline earth salts of violuric acid (5-isonitroso barbituric acid) have characteristic colors and different solubilities of the salts in dimethyl formamide.

It may be concluded from this survey of chemical methods available for the analysis of serum sodium that there exist a number of simple procedures which are sufficiently accurate for clinical purposes and which are rapid and inexpensive to perform.

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B. Gravimetric Method for the Measurement of Sodium in Serum

F. WILLIAM SUNDERMAN, M.D., Ph.D.

INTRODUCTION

A modification of the Barber and Kolthoff method[1] is herewith included for the estimation of sodium in biological fluids. This gravimetric method is our preference for reference purposes.

PRINCIPLE

After a preliminary ashing of serum, sodium is precipitated and weighed as uranyl zinc sodium acetate, $(UO_2)_3\,Zn\,Na(CH_3COO)_9 \cdot 6H_2O$.

REAGENTS

1. *Uranyl Zinc Acetate Reagent. Solution A.* To 38.5 grams of uranyl acetate are added about 200 ml. of distilled water and 7 ml. of glacial acetic acid. The solid components are dissolved by gentle heating with stirring, then cooled and diluted to 250 ml. in a volumetric flask.

Solution B. To 115.4 grams of zinc acetate are added about 200 ml. of distilled water and 3.5 ml. of glacial acetic acid. The solid components are dissolved by gentle heating with stirring, then cooled and diluted to 250 ml. in a volumetric flask.

The two solutions (A and B) are then heated and mixed while hot. The mixture is placed in an incubator at 38° C. for forty-eight hours and is occasionally shaken. Owing to sodium impurities in the reagents, a precipitate of uranyl zinc sodium acetate forms and settles out. If no precipitate appears, 0.2 gram of precipitated uranyl zinc sodium acetate is added in order to saturate the solution with this triple salt. About one hour before using, the solution is removed from the incubator and allowed to come to room temperature. The bottle is shaken, and as much solution as is needed is filtered immediately before using.

2. *Alcohol Wash Solution.* Ninety-five per cent ethyl alcohol is saturated with uranium zinc sodium acetate precipitate. The alcohol must be 95 per cent and should be prepared from absolute alcohol. When not being used, this solution is stored in the incubator at 38° C. About one hour before use, it is brought to room temperature with occasional shaking. It is filtered just before using as a wash solution.

3. *Uranyl Zinc Sodium Acetate Precipitate.* A small amount of the triple salt may be prepared by adding 15 ml. of the uranyl zinc acetate reagent to 1 ml. of a 5 per cent solution of sodium chloride. Five ml. of 95 per cent alcohol are added in small portions. The mixture is filtered with suction in a Gooch crucible and the precipitate is washed four times with 5-ml. portions of alcohol, followed by four washings with 5 ml. portions of ether, sucking dry after each addition of alcohol or ether.

4. *Ethyl alcohol, 95 per cent.* This solution is prepared from absolute alcohol.

5. *Ethyl Ether.* Reagent grade.

6. *Sulfuric Acid.* Four normal.

7. *Ferric Sulfate.* This solution is prepared by dissolving 1.25 gram of ferric sulfate in 100 ml. of distilled water.

8. *Standard Salt Solution.* Using recrystallized and dried NaCl, KCl, $MgSO_4$ and $CaSO_4$, a salt solution is prepared which will have approximately the same base concentration as serum.

7.891 gm./L. NaCl = 135.0 mEq./L. Na
0.298 gm./L. KCl = 4.0 mEq./L. K
0.340 gm./L. $CaSO_4$ = 5.0 mEq./L. Ca
0.120 gm./L. $MgSO_4$ = 2.0 mEq./L. Mg
146.0 mEq./L.
Total Base

The salts are weighed as shown and dissolved in distilled water, diluting to the mark in a liter volumetric flask.

PROCEDURE

1. One ml. of serum is placed in a 30 ml. silica beaker or Vycor crucible. One ml. of 4N H_2SO_4 and one drop of ferric sulfate solution are added. The ferric sulfate is added to precipitate the phosphate present. One ml. of the standard salt solution is carried through the procedure in the same manner as the unknown. One ml of H_2O is carried along as a blank. Analyses of serum, salt solution, and blank are made in duplicate or triplicate. The crucibles are placed on an electric sandbath under the hood until the mixture is dry and no more SO_2 fumes are given off (about three hours). The crucibles are then placed in an electric furnace, gradually raising the temperature to 550° C. This heat is maintained for one hour or until ashing is complete. There will be a red discolorization in the salt solution owing to excess iron. The crucibles are allowed to cool to room temperature and the ash is transferred to a graduated 25 ml centrifuge tube with four or five distilled water washings. The solution is diluted to the mark and mixed. After centrifuging for five minutes, 20 ml. of the supernatant fluid are placed in a 50 ml. pyrex beaker and evaporated to dryness in an oven at 100°C.

2. Porous, glass Gooch filter crucibles

of 15-ml. capacity are placed in filter funnels over 500-ml. filter flasks.* They are prepared by washing once with water, three times with 2 ml. alcoholic wash solution saturated with uranyl zinc sodium acetate, and three times with 2 ml. ether. Air is suctioned through the Gooch crucibles for ten minutes, following which they are placed in the desiccator for twenty minutes and weighed.

3. To the ashed serum, 10 ml. of uranyl zinc acetate are added, and the whole is well mixed and allowed to stand for thirty minutes in water at room temperature. The contents are then filtered through a prepared crucible. The beaker and precipitate are then washed ten times with 2 ml. of the alcoholic wash solution. The precipitate in the crucible is then washed three times with 2 ml. of ether. Air is now drawn through the crucible for ten minutes until the ether is removed. The crucible is allowed to stand in the desiccator for twenty minutes, and then is weighed.

4. Filtration must be carried out at approximately the same temperature as precipitation in order to avoid changes in solubility of the uranyl zinc sodium acetate.

CALCULATIONS

The salt: $(UO_2)_3\,Zn\,Na\,(CH_3COO)_9 \cdot 6H_2O$, is formed.

$$\text{gm. of Na} = \text{gm. ppt.} \times 0.01495$$

$$\text{mEq. of Na} = \text{gm. ppt.} \times 0.65$$

$$\text{Na mEq./L.} = \left(\text{gm. ppt.} \times 0.65 \times \frac{25}{20} \times 1000\right) \text{ or}$$

$$= \text{gm. ppt.} \times 812.5$$

Correction is made for the blank.

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*It is advisable to have the crucibles thoroughly cleaned with HC1 before-hand and rinsed with distilled water.

C. Colorimetric Method for the Measurement of Sodium in Serum

BERNARD J. KATCHMAN, Ph.D., and ROBERT E. ZIPF, M.D.

INTRODUCTION

The methods available for the analysis of sodium in biologic fluids have been reviewed recently by Sunderman[1] and by Henry[2]. The method reported below is that of Albanese and Lein[3], as modified by Henry[2].

PRINCIPLE

The sodium of serum is precipitated from a trichloroacetic acid filtrate as sodium uranyl zinc acetate, $(UO_2)_3Zn\,Na(CH_3COO)_9 \cdot 6H_2O$. The precipitate is dissolved in water and determined photometrically.

REAGENTS

1. *Uranyl Zinc Acetate Reagent.* Ten grams of uranyl acetate are dissolved in 50 ml. of boiling water containing 2 ml. of glacial acetic acid. In another flask, 30 grams of zinc acetate are dissolved in 50 ml. of boiling water containing 1 ml. of glacial acetic acid. The boiling solutions are mixed and reheated to boiling. The mixture is left at room temperature over night and then filtered. The filtrate is mixed with an equal volume of 95 per cent ethanol, and the resultant solution is refrigerated at 4° C. for forty-eight hours, after which it is filtered. The reagent is stable at room temperature.
2. *Ethanol,* 95 per cent (V/V)..
3. *Trichloroacetic Acid,* 10 per cent (W/V).

STANDARD SOLUTIONS

1. *Sodium Standard* (0.64 mg. of sodium per ml.) one hundred-sixty-two mg. of ovendried (100° C.) reagent grade sodium chloride are dissolved and made up to 100 ml. with distilled water.

PROCEDURE

1. An aliquot of serum, 0.5 ml. is pipetted carefully into a small test tube, and 2.0 ml. of 10 per cent trichloroacetic acid are added. The tube is carefully mixed, and after five minutes, is centrifuged
2. Into conical, 15-ml. centrifuge tubes are pipetted the following:
   a. *Unknown sample,* 0.50 ml. of trichloroacetic acid supernatant,
   b. *Sodium standard,* 0.50 ml. standard solution (0.32 mg. Na),
   c. *Blank,* 0.50 ml. distilled water.
3. 1 ml. of the uranyl zinc acetate reagent is added to each tube and mixed. All tubes are refrigerated for one hour and then centrifuged. The supernatants are decanted, and the tubes are drained by inversion on absorbent paper.
4. The walls of the tubes are rinsed with 2.0 ml. of 95 per cent ethanol. The tubes are shaken to disperse the precipitate in the alcohol. The tubes are centrifuged and drained.
5. The precipitate is dissolved with 5.0 ml. of distilled water. If turbid, the solution is centrifuged.
6. All solutions are read against water at 430 mμ. The blank should have very little or no absorbance.

DISCUSSION

Figure 1 is a plot of optical density at 430 mμ versus concentration of sodium standard solution. A straight line may be obtained over the range 0.128 to 0.448 mg. of sodium. Note that the straight line does not extrapolate to zero optical density (Curve A). Therefore, a simple ratio of $\frac{\text{O.D Sample}}{\text{O.D. Standard}}$ x Concentration of Standard, if used for calculation, inherently produces an error the magnitude of which can be seen as a horizontal line drawn between Curve A and Curve B. Samples with optical densities different from that of the standard produce progressively greater errors, depending upon the magnitude of difference between the optical den-

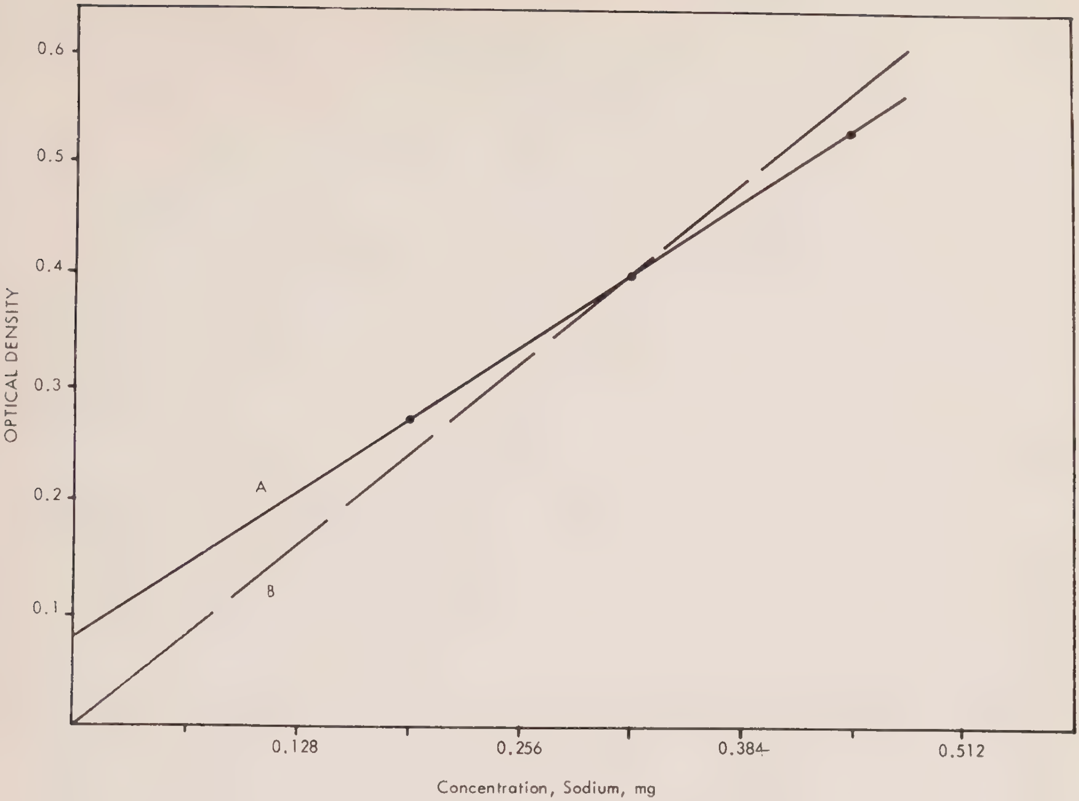

Fig. 1. Calibration Curve for the Colorimetric Measurement of Serum Sodium.

sity of the standard and that of the sample; samples with optical densities less than the standard err on the high side, and samples with optical densities greater than the standard err on the low side. If the standard (in this case) has an optical density of 0.405, then a sample whose optical density lies between 0.370 and 0.430 mμ will be in error by ± 2 per cent. Within this narrow range of values, the simple ratio formula (seen under Calculations) may be used. In order to avoid this mathematical error, a standard curve may be used to obtain the concentration of the sample.

The Beckman DU spectrophotometer (slit width 0.025 mm.) or the Beckman DK recording spectrophotometer (slit width 0.15 mm.) give identical results. The sensitivity with the Beckman instruments is nearly twice that reported by Albanese and Lein using the Klett-Summerson filter photometer with filter S-42.

CALCULATIONS

1. *Simple Ratio Formula*

$$\text{mg. Na/ml.} - \frac{\text{O.D. Sample}}{\text{O.D. Standard}} \text{ x Concentration x Dilution}$$

$$= \frac{\text{O.D. Sample}}{\text{O.D. Standard}} \text{ x } 0.32 \text{ mg. Na x } 1000$$

$$\frac{\text{O.D. Sample}}{\text{O.D. Standard}} \text{ x } 320$$

$$\text{mEq. Na/l.} = \frac{\text{O.D. Sample}}{\text{O.D. Standard}} \text{ x } 139$$

2. *Standard Curve*

mg. Na/ml. = Concentration of sample, mg. x 1000

mEq. Na/l. = Concentration of sample, mg. x 435.

SOURCES OF ERROR

Theoretically, there are two main sources of error. One is the slight solubility of the sodium zinc uranyl acetate precipitate. The other arises from the use of reagents that are saturated with respect to sodium zinc uranyl acetate and in which the solubility of the sodium zinc uranyl acetate precipitate may be altered by inadequate control of laboratory procedures. These errors may be minimized if the procedure is standardized and if, especially, the technique for washing the precipitate is rigidly controlled and duplicated for both control and unknown samples.

Henry[2] reports reproductibility of the procedure to be about ± 5 per cent. In checking the procedure in our laboratory, we find that serum samples can be analyzed to ± 3 per cent and urine samples to ± 5 per cent.

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The Chemical Measurement of Potassium in Biological Fluids

A. General Considerations of the Cobalti-nitrite Method for the Measurement of Potassium

ALBERT CANNON, M.D., and RICHARD GADSDEN, Ph.D.

INTRODUCTION

Since variations from the normal levels of the concentration of potassium in plasma of humans are accompanied by profound metabolic derangements, the importance of being able to obtain fast and accurate estimations of plasma potassium levels is readily appreciated. Once this was realized, many attempts were made to satisfy this demand by the development of new procedures, as well as modifications of the existing ones. It is the purpose of this paper to trace the evolutionary development of one of the satisfactory chemical procedures still used. Mention will be made of other chemical procedures, both old and recent.

The word "potassium" is derived from the English "potash," and its chemical symbol, "K," comes from the Latin "kalium." This element was first discovered by Davy in 1807, who obtained it from caustic potash (KOH). It was the first metal to be isolated by electrodeposition[18].

Potassium is the eighth most abundant element forming the earth's crust, and it comprises approximately 2.0 per cent of the crust[4, 18]. It is found in the alumina silicate minerals, such as feldspar and mica, which are constituents of granite. Most of its salts are insoluble, therefore the element is separated artifically from them with difficulty. However, under certain conditions, potassium salts can be concentrated from the beds of ancient oceans, which are found in Prussian Saxony and the western United States[18]. With weathering of these granite constituents, sodium and potassium complexes are released. The sodium complexes are insoluble and are leached into rivers and carried to sea. The potassium is absorbed by clay. It is freed from the soil by the action of growing vegetation and incorporated into its metabolism as a vital element[33].

Potassium also occupies an important

place in the physiology of animals. It is the principle cation of the intracellular fluid, and along with sodium, is one of the most important elements in the extracellular body fluid. The variations in both intracellular and extracellular potassium levels are influenced by changes in the acid base balance of the body and by hormones of the adrenal cortex. Marked variations from normal levels are accompanied by severe clinical symptoms and even death. The normal distribution of potassium is given in the following table[16]:

FLUID OR TISSUE	MG./100 ML. OR 100 GM.	
Whole Blood	200	(50 mEq./L.)
Plasma	20	(5 mEq./L.)
Cells	440	(112 mEq./L.)
Muscle Tissue	250-400	
Nerve Tissue	530	

The cobalti-nitrite method for the estimation of potassium in biological fluids, with its various modifications, seems to have stood the test of time, in spite of theoretical and practical drawbacks which will be discussed in detail. The method of Adie and Wood[2] which was adapted for serum by Kramer and Tisdall(26) and further modified by Lochhead and Purcell[27] appears to be a reliable procedure for the measurement of potassium in serum. A modification by Gadsden[14] has shortened the procedure so that the test can be used routinely for clinical purposes.

DeKoninck, in 1881, probably was the first to make use of the reaction between cobalti-nitrite and potassium as a test for the latter, and the reagent was called by his name[9]. However, he used it for qualitative purposes only, and he found that it was much more sensitive than platinum chloride for the detection of potassium. An immediate, yellow precipitation was obtained when the mixture of sodium nitrite, cobalt chloride, and acetic acid were added to one part of KC1 and one thousand parts of water. DeKoninck also found that ammonia gave a similar reaction, but that sodium, magnesium, calcium, iron, and zinc salts, as well as sulfate, nitrate, and chloride ions, were not precipitated. Bowser quotes Fresenius, writing in Well's *Qualitative Analysis,* 16th edition, as calling attention to the fact that in concentrated solutions, precipitation occurs immediately, but that in dilute solutions, it occurs only gradually. He also noted that ammonium salts gave a reaction similar to that of potassium, but only when concentrated solutions were used[9]. Bowser also quotes Prescott and Johnson, writing in *Qualitative Chemical Analysis,* 6th edition, as calling attention to the fact that the precipitate is sparingly soluble in water, insoluble in alcohol and in solutions of potassium salts[9]. These observations were used later in modifications of the original procedures in order to adapt it to determinations of serum potassium and to increase its accuracy.

Adie and Wood, in 1900, and Drushel, in 1907, published methods for potassium determinations using cobalti-nitrite. In the method of Adie and Wood, the potassium was precipitated with sodium cobalti-nitrite[2]. The reactions involved in the precipitation of dipotassium sodium cobalti-nitrite were given as follows:

1. $21\ Co(C_2H_3O_2) \bullet 4\ H_2O + 14\ NaNO_2 + 4\ H(C_2H_3O_2) =$
   $Na_6\ Co_2\ (NO_2)_{12} \bullet H_2O + 8\ Na(C_2H_3O_2) + 2\ NO + 6H_2O$
2. $Na_6\ Co_2\ (NO_2)_{12} \bullet H_2O + 2K_2SO_4 =$
   $2\ K_2NaCo\ (NO_2)_6 \bullet H_2O + 2\ Na_2SO_4$
   yellow insoluble precipitate

When the precipitate was treated directly with permanganate, the following reaction occurred:

$$10(K_2NaCo\ (NO_2)_6 \bullet H_2O) + 11\ K_2Mn_2O_8 + 58\ H_2SO_4 =$$
$$21\ K_2SO_4 + 5\ Na_2SO_4 + 10\ CoSO_4 + 22\ MnSO_4 + 60\ HNO_3 + 38\ H_2O$$

After standing overnight, the precipitate was filtered on a Gooch filter and washed with acetic acid. The cobalt was precipitated by boiling with NaOH. The filtered solution was then titrated with $KMnO_4$. One ml. of the N/10 $KMnO_4$ is equal to 0.0007858 grams of K_2O. Adie and Wood isolated and analyzed the potassium sodium cobalti-nitrite compound and showed that the precipitate had a constant composition represented by the formulae $K_2Na[CO(NO_2)_6] \bullet H_2O$. They also determined its solubility to be less one part in 20,000. However, the currently accepted value for solubility is 0.07 grams in 100 ml. of water 25° C., thus indicating greater solubility[18].

The method of Drushel differed in that the dipotassium sodium cobaltinitrite was evaporated in a steam bath, filtered on asbestos and oxidized with hot standard $KMnO_4$[9]. The excess of permanganate was bleached by an excess of standard oxalic acid, and the solution back-titrated with permanganate. Bowser's modification of these two methods gave excellent results for potassium assay of soil, as long as the laborious procedure was rigidly followed. It was considered superior to the platinichloride method previously used, because the potassium could be present as the chloride, sulfate, or nitrate. It was also much more economical.

In 1910, Shedd, a soil chemist, modified Drushel's method by concentrating the unknown solution by evaporation before an excess of reagent was added[34]. This apparently was not considered important in serum work until 1953, when dilution of the sample with water was shown to be a source of incomplete precipitation of potassium. Shedd also called attention to the increase in solubility of the precipitate when heated with acetic acid, and he showed the necessity for keeping the sodium cobaltinitrite reagent in the dark to avoid precipitation. He also recognized the complexity of incomplete precipitation and suggested the addition of the reagent in two portions to precipitate more completely the potassium by maintaining an excess of fresh reagent in the solution.

In 1911, Bowser called attention to the sensitiveness to external conditions of the older platinic chloride method of potassium and the superiority of the method of Adie and Wood, which he modified, so that small amounts of potassium could be estimated by titrimetric determination[10]. He found that a good precipitate could be obtained by adding strong acetic acid after the addition of the cobalti-nitrite reagent. However, when glacial acetic acid was added, the precipitation was incomplete, and the granules were not uniform. Also, rapid decomposition of the precipitate and reagent occurred. If, however, equal portions of glacial acetic acid and alcohol were added after the reagent, then the precipitate was rapid, complete, and the granules were uniform and coarse.

MacDougall, in 1912, described a modification of the existing methods by having the oxidization of the nitrite by the permanganate take place in an alkaline solution[29]. The permanganate is first reduced to manganese dioxide which interacts slowly with the oxalic acid, subsequently added, since the reaction mixture is so dilute.

Apparently, the first attempt to determine potassium, using small amounts of blood, was made by Hamburger in 1915[15]. He devised a centrifuge tube in which the lower end was drawn out into a capillary tube and graduated. The volume of precipitate was measured in this graduated capillary portion and compared to a known standard. The time for precipitation was sixteen hours.

In 1918, Greenwald described a composite method for the determination of potassium, sodium, calcium, and phosphate in blood, but his method required 90 grams of blood[24].

In 1918, Clausen published a method of estimating potassium in small quantities of blood, using the modified cobalti-nitrite method of Adie and Wood[11]. Owing to the small amounts of potassium involved, a dilute permanganate solution was used, but the procedure called for oxidization of the dipotassium sodium cobalti-nitrite in acid solution at boiling temperature, which caused an appreciable decomposition of the dilute permanganate.

In order to eliminate this error, an abandoned step in the original procedure was used. The precipitate of potassium sodium cobalti-nitrite was heated with dilute NaOH, which changed all the nitrite groups to sodium and potassium nitrite. The cobalt was precipitated as insoluble $Co(OH)_3$ and was filtered. The nitrites could then be estimated by titration with dilute potassium permanganate. Clausen found that the calcium, magnesium, iron, and phosphates of blood did not interfere with the estimation of potassium. He removed the organic material by a "wet ashing process," using sulfuric and nitric acid. The time allowed for precipitation and the many laborious steps, including washing the asbestos filter six times, did not result in a practical procedure for clinical laboratories. However, the way was paved for others to develop such a test which would furnish a practical, clinical method for the estimation of potassium.

It was realized that a most desirable feature of any method for estimation of serum potassium should include the ability to precipitate this cation directly from serum without previous treatment of the serum. As early as 1871, Pribrom had shown that calcium could be precipitated from alkaline serum and measured quantitatively[26]. In 1917, Marbiott and Haessler had also shown that inorganic phosphorus and magnesium could also be directly precipitated from serum in a satisfactory measurable form [26]. In 1921, Kramer, who, in 1920, had modified the titrimetric cobalti-nitrite method and investigated the effects of inorganic phosphates on the method with Tisdall, showed that potassium could likewise be precipitated from serum directly, using an excess of sodium cobalti-nitrite without the removal of organic material[26]. This was accomplished by adjusting the pH of the serum so that the proteins remained in solution. Their method called for the addition to serum of an excess of sodium cobalti-nitrite at a pH of 5.7, which is more alkaline than the isolectric point of the serum proteins. The resulting precipitate was centrifuged and washed with water, then titrated with permanganate as in Kramer's original procedure.

In a study of the inorganic elements of blood plasma in disease, Briggs, in 1923, modified the Kramer and Tisdall procedure by filtering the fine, granular precipitate on paper pulp[7]. The subsequent decomposition of the precipitate with sodium hydroxide released reducing substances from the paper which interferred with the titration by permanganate. Briggs was able to measure the nitrites successfully by employing the nitrite color reaction of Geiss, in which sulfanilic acid and l-naphthylamine were used[12]. The method, however, was of limited value as a routine procedure owing to the use of a special filter and the long duration of initial precipitation (four hours).

Studies on the inorganic composition of blood from dogs, by Kerr in 1926, using a protein-free filtrate prepared by treating the blood with trichloroacetic acid, failed to agree with the results obtained by the Kramer–Tisdall method when duplicate samples were analyzed[23]. Kerr postulated that the serum proteins occluded some of the potassium cobalti-nitrite and also prevented oxidation by permanganate. During the same year, Fiske and Litarczek developed a method for the analysis of potassium in serum[13]. They employed the cobalti-nitrite precipitation, followed by ashing of the precipitate and reprecipitation of the potassium with alcoholic acid tartrate, which is titratable by a standard alkali.

Breh and Gaebler, in 1930, presented a study on the determination of potassium in canine serum in which they experienced difficulties in applying the Kramer-Tisdall method, owing to the presence of protein in the potassium cobalti-nitrite precipitate[6]. A further complication was turbidity owing to suspended fat. To avoid ashing, they precipitated the potassium directly from a modified, tungstic-acid filtrate as potassium silver cobalti-nitrite. This compound is far more insoluble than the corresponding potassium sodium salt. For the quantitive estimation of potassium in the precipitate, Breh and Gaebler used a colorimetric method based on the blue color produced by the reaction between salts of cobalt and ammonium thiocyanate in an alcoholic solution. Since the intensity of the color varies with temperature and concentration of alcohol, a calibration curve must be constructed for each set of determinations. The cobalti-nitrite precipitate may also be analyzed by the gasometric method of Kramer and Gittleman[25].

In 1931, Jacobs and Hoffman reviewed the methods of potassium estimation in serum and urine employing cobalti-nitrite[21]. They were of the opinion that "from a theoretical point of view, those methods which determined directly a stable constituent of the precipitate were preferable to those which depended upon the activity of the unstable nitrite radical." The methods so far described which embodied this principle were those of Fiske and Litarczek, and of Breh and Gaebler. Jacobs and Hoffman described a relatively simple method which embodied the theoretical advantages to which they had called attention. Their method was based on the formation of a stable, colored compound when ferrocyanide was added to a mixture of a cobaltous salt and choline hydrochloride. An emerald-green color developed to maximum intensity in a few minutes and was found to be sensitive to minute amounts of choline, cobalt and ferrocyanide, so that, in the presence of an excess of choline and ferrocyanide, it could be used to determine colorimetrically small variations in the concentration of cobalt.

The relative simplicity of this colorimetric method, requiring only two accurate measurements and employing the direct precipitation of potassium from serum, produced, for the first time, a method which could be used clinically.

In 1932, Peters and Van Slyke published a review of the various cobaltinitrite methods[31]. They called attention to the attractive possibilities which the basic method had and to the controversey which had raged during the preceeding thirty years since the method was described by Adie and Wood. As had been shown, the precipitate of dipotassium sodium cobalti-nitrite had been analyzed in many various ways, including, gravimetric, gasometric, titrimetric, and colorimetric means. The latter two means afforded ideal micro procedures, though there was the apparent inability to find conditions under which this precipitate could be formed which would give it a constant composition. However, Van Rysselberger's work, published in 1931, appeared to have placed the method on a much more secure basis[40].

Apparently, variation in weight of the dipotassium cobalt precipitate was due, in part, to the water of hydration. However, according to Bonneau, the precipitate had a constant composition only so long as the ratio of sodium to potassium in the solution exceeded twenty-two[8]. If there were more than $\frac{1}{22}$ as much potassium as sodium in the solution from which precipitation occurs, then the composition of the precipitate tended to be of the tripotassium cobalt salt with the following formula: $K_3Co(NO_2)_6$. When the sodium potassium was lowered to one, then the entire precipitate was composed of the tripotassium cobalt salt.

Both Bonneau and Van Rysselberger recommended that control analysis be done, employing solutions of the approximate sodium-potassium ratio that was expected to appear in the unknown. By this method, the latter stated that the error of potassium could be reduced to 1.3 per cent. A review of the various sources of error in the methods was made by Barry and Rowland in 1953[5]. They called attention to the need of using standards similar in composition, particularly as far as the Na:K ratio was concerned, and the employment of these standards with each set of determinations. They described three main sources of error when analyzing solutions containing small concentrations of potassium. These were as follows:

1. Incomplete precipitation of potassium due to the solubility of the dipotassium cobalt salt in the solution from which precipitation occurs. Therefore, they advocated not diluting the serum with water, either before or after the reagent is added. When this was done, they found that 15 to 20 per cent of the potassium remained unprecipitated.

2. The duration and the temperature both affect the amounts of potassium sodium cobalt salt precipitated. They recommended carrying out the analysis at a constant temperature and increasing the time of precipitation to two hours. The latter is a disadvantage in any clinical chemical procedure.

3. There can be significant loss of precipitate in washing if ethanol is not used. Barry and Rowland recommended a 30 per cent mixture for the first washing to prevent precipitation of protein and 70 per cent mixture for subsequent washings.

They also recommended the rapid addition of the cobalti-nitrite to the serum so that a higher Na:K ratio could be maintained at the time of precipitation, which caused a larger amount of precipitation.

Using these improvements and the Jacobs-Hoffman photometric method of determining cobalt, Barry and Rowland were able to analyze duplicate samples in the 20 mg. range with a coefficient of variation of 1 per cent. In addition to the cobalti-nitrite method, with its many modifications, there should also be mentioned the cholorpatinate precipitation method, which is considered the most accurate of the clinical methods, but which requires ashing the sample[7].

In addition, mention should be made of the tetraphenylboron method of Sunderman, in which the turbidity of the potassium tetraphenylboron solution is measured[38]. This method is relatively simple and inexpensive, and it agrees excellently with flame photometry[14]. More complicated methods employing neutron activation[34] and spectrographic analysis[36] have also been described.

A detailed review of the evolution and development of a practical cobaltinitrite chemical method for the determination of serum potassium has been presented. The various modifications and refinements of Adie and Wood's original procedure of 1900 have been described.

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B. Cobalti-nitrite Method for the Measurement of Potassium in Serum[13]

RICHARD H. GADSDEN, Ph.D. and ALBERT CANNON, M.D.

PRINCIPLE

Potassium is precipitated from serum as the insoluble potassium sodium cobalti-nitrite. The precipitate is carried through several washes in order to eliminate interfering substances. Finally, it is dissolved and reacted with phenol reagent (Folin-Ciocalteau) in the presence of glycine in an alkaline medium. The reduction of the phenol reagent is a result of the cobalt of the cobalti-nitrite and is proportional to the potassium precipitated as the salt.

The reactions are as follows:

$$K^+ + \underset{\text{(Serum)}}{Na_3[Co(NO_2)_6]} \xrightarrow[\text{Room Temp.}]{\text{NaAc}} K_2Na[Co(NO_2)_6]$$

$$K_2Na[Co(NO_2)_6] + H_2O \xrightarrow[\text{Room Temp.}]{} \downarrow K_2Na[Co(NO_2)_6] \cdot H_2O$$

$$K_2Na[Co(NO_2)_6] + H_3PMo_{12}O_{14} \xrightarrow[\text{(Glycine)}]{(Na_2CO_3)} \text{Molybdenum Blue} + Co^{++}$$

REAGENTS

1. *Potassium Standard, STOCK* (0.4 mg. per ml.) Crystalline K_2SO_4 is dried overnight at 100° C. Exactly 892 mg. K_2SO_4 are dissolved in water and diluted to 1 L.

2. *Potassium Standard, Working* (0.2 mg. per ml.) The STOCK standard is diluted 1:1 with water.

3. *Sodium Cobaltinitrite* (K-precipitating reagent): A. Twenty-five grams $Co(NO_3)_2$ (or 42.25 grams $Co(NO_3)_2 \cdot 6H_2O$) are dissolved in 500 ml. of water to which is added 12.5 ml. of glacial acetic acid. B. One hundred and twenty grams of $NaNO_2$ are dissolved in 180 ml. water. Two hundred and ten ml. of solution B are added to solution A. The mixture is aerated in a fume hood until all fumes of nitrous oxide have been driven off, and the mixture is filtered. The reagent is stable for four to six weeks when stored under refrigeration. This reagent must be filtered before use.

4. *Sodium Acetate*, one-half saturated: One hundred and thirty grams $Na\text{-}AC \cdot 3H_2O$ (or 79 grams Na-Ac anhydrous) are added to 100 ml. water preheated to 45 to 50° C. The solution is shaken in a 37° C. incubator overnight. It is allowed to cool to room temperature, permitting excess Na-Ac to precipitate. After filtration, the saturated solution is diluted with an equal volume of water.

5. *Wash Solution* (saturated with potassium-sodium cobalti-nitrite). Seventy mg. of K_2SO_4 are dissolved in 100 ml. of water. To the solution are added, in sequence, 25 ml. one-half saturated solution of Na-Ac and 12.5 ml. of filtered Na-cobalti-nitrite reagent. The mixture is allowed to stand undisturbed for thirty to forty-five minutes and is filtered by suction. The precipitate is washed twice with 70 per cent (v/v) and once with 95 per cent (v/v) ethanol. Ten per cent ethanol is saturated with the precipitate (the amount of K-cobalti-nitrite prepared will adequately saturate 200 ml. of 10 per cent of ethanol leaving a slight excess). Placing the mixture on a magnetic stirrer for four hours facilitates solution. The solution is filtered by suction or through quantitative filter paper (Whatman No. 40) before use.

6. *Glycine,* 7.5 per cent (w/v) solution.

7. *Sodium Carbonate,* 25 per cent (w/v).

8. *Phenol Reagent.* One hundred grams of sodium tungstate, $Na_2WO_4 \bullet 2H_2O$ and 25 gm. of sodium molybdate, $Na_3MoO_4 \bullet 2H_2O$ are dissolved in 700 ml. of water. To this solution are added 50 ml. of 85 per cent H_3PO_4 and 100 ml. of concentrated HCl. The solution is boiled gently for ten hours in a flask connected to a reflux condenser. After cooling, 150 gm. of lithium sulfate and a few drops of bromine are added. The mixture is then boiled to remove excess bromine and, after cooling, is diluted to 1 L. with water. The reagent should have no greenish tint. It should be protected from dust.

9. *Phenol Reagent, Working.* One volume of stock reagent is diluted by the addition of two volumes of water. This solution is prepared fresh before use.

PROCEDURE

Into heavy-walled centrifuge tubes are pipetted:

1. Standard: 0.2 ml. working standard.
   Unknowns: 0.2 ml. serum or plasma.
   To all tubes are added 0.2 ml. of one-half saturated Na-Ac.

2. With the centrifuge tube in a Vortex mixer 0.5 ml. of Na cobalti-nitrite reagent is added dropwise. All tubes are treated in like manner, and are allowed to stand at room temperature for exactly forty-five minutes.

3. To all tubes are added 1 ml. water, and the contents are mixed with a Vortex mixer. The tubes are centrifuged at high speed for ten to fifteen minutes.

4. The supernatant is gently decanted and allowed to drain inverted on an absorbant surface for ten to fifteen minutes. The excess fluid is wiped from the lip of the tubes.

5. Without disturbing the precipitate, 0.5 ml. of wash solution is added to all of the tubes, and the tubes are centrifuged for ten to fifteen minutes. The supernatant is decanted, and the tubes are inverted and drained ten to fifteen minutes. The excess fluid is wiped from the lip of the tubes.

6. One ml. of 70 per cent ethanol is added to all tubes. With thin glass rods, the precipitate is dislodged from the bottoms of the tubes and dispersed. The glass rods are rinsed with an additional 3 ml. of 70 per cent ethanol into the corresponding centrifuge tubes. Each tube is swirled on a Vortex mixer, and centrifuged for five to ten minutes. The supernatant is decanted and drained as

before for five to ten minutes, and excess fluid is wiped from the lip of the tubes.

7. Four ml. of 70 per cent ethanol are added to each tube, and the tube is swirled on a Vortex mixer. Centrifugation and drainage are repeated as in step 6. (*Note*: At this stage, the precipitates may be preserved by the addition of a drop of water and stoppering the tube.)

8. To each tube and Blank tube are added 2 ml. of water. The precipitate is dispersed with a glass rod, and the rod is left *in situ*. All tubes are placed in a boiling water bath (gentle stirring will facilitate dissolving of the precipitate). When the precipitate is completely dissolved (a small amount of white precipitate may not dissolve, but this is of no consequence), the following are added in order to the solution while still hot: 1 ml. of 7.5 glycine and 1 ml. 25 per cent Na-carbonate. The contents are mixed thoroughly.

9. To all tubes are added 1 ml. of working phenol reagent. The contents are mixed thoroughly. The tubes are incubated in a 37° water bath for fifteen minutes and cooled to room temperature. The glass rod is rinsed with small amounts of water, and the contents are diluted to a final volume of 7 ml.

10. Absorbancies (OD) of standards and unknowns are measured against a blank set at 0 O.D. at 660 mM.

Calculation (reduction of phenol reagent is proportional to K concentration):

$$\frac{\text{OD Unknown}}{\text{OD Standard}} \begin{cases} \times 20 = \text{mg. K per 100 ml.} \\ \times 5.13 = \text{mEq. K per L.} \end{cases}$$

Normal values for serum potassium as determined by different workers using various methods of analysis are tabulated in Table I. Regardless of the method, it is generally agreed that the normal range for human adults is 3.3 to 5.5 mEq. per L. The normal values as determined by various workers for children are shown in Table II. These values are cited to emphasize the higher normal limits of serum potassium in children.

Hyperkalemia[5, 11] may occur as a result of increased potassium intake; impaired renal function, untreated (oliguria, anuria); tissue breakdown (trauma and perhaps necrosis); anoxia; contraction of extracellular volume by dehydration. It is a common finding in adrenal-cortical insufficiency (Addison's disease). A most prevalent cause is the too-rapid administration of solution containing high potassium concentration to dehydrated patients with low urine volume output and/or with renal function impairment.

Hypokalemia is a more frequent clinical occurrence and manifests itself in: excess loss of potassium from the body (diuresis, vomiting, diarrhea, fistulas, tube drainage from stomach and/or intestines, renal tubular acidosis); transfer of excessive amounts of potassium from extracellular to intracellular fluids (as in administration of glucose, insulin, or epinephrine); dilution of extracellular fluids by administration of fluids having low or no potassium content; inadequate potassium intake usually in association with the above; increased adrenal-cortical activity. Hypokalemia of familial periodic paralysis is a relatively rare phenomenon. The flaccid paralysis is of the extremities, whereas, that associated with hypokalemia of diabetic acidosis is of the respiratory musculature.

TABLE I. NORMAL SERUM POTASSIUM VALUES FOR HUMAN ADULTS AS ESTABLISHED BY INDIVIDUAL ANALYTICAL METHODS

METHOD	VALUE (Range–mEq./L.)	REFERENCE
Chloroplatinate	3.4–6.5	10
	3.1–5.3	9
	3.0–7.6	20
	3.8–4.3	8
Cobalti-nitrite	4.8–5.1	12
	4.0–5.4	1
	4.6–5.3	22
Tetraphenylboron	3.8–5.4	3
	4.0–6.0	17
Flame Photometry	3.7–5.1	24
	3.6–6.2	14
	3.6–6.2	21
	3.6–4.9	18
	3.5–5.6	15
	3.2–5.6	6
	3.5–5.5	4

TABLE II. SERUM POTASSIUM—NORMAL VALUES FOR CHILDREN

RANGE (mEq./L.)	AGE	REFERENCE
3.7–5.6	Children	2
3.2–5.9	Infants	6
3.2–4.3	Children	
4.1–7.6	Prematures	16
3.9–7.1	5 days	19
3.8–5.5	Newborn	7
4.0–6.5	3 months	
3.8–6.1	6 months	
4.0–6.1	9 months	
3.8–5.9	12 months	
2.7–5.7	3-6 years	
4.0–7.0	First 10 days—not uncommon	23
4.0–5.8	For infants and children	23

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C. Platinic Chloride and Tetraphenylboron Methods for the Measurement of Potassium in Serum

F. WILLIAM SUNDERMAN, JR., M.D., and F. WILLIAM SUNDERMAN, M.D.

INTRODUCTION

Measurements of potassium are described by means of (a) a reference method, and (b) a rapid turbidimetric method. The platinic chloride method has been selected as the reference procedure because of its accuracy and dependability. The tetraphenylboron method has been chosen for the second procedure because of its simplicity and adaptability to routine clinical analysis.

PLATINIC CHLORIDE METHOD FOR SERUM POTASSIUM[1,2,4,6]

PRINCIPLE

Serum is ashed in a muffle furnace, and potassium is precipitated as potassium chloroplatinate. The chloroplatinate is treated with iodide to yield potassium iodoplatinate. Finally, the red-brown iodoplatinate is reduced to yellow iodoplatinite by titration with standard thiosulfate.

$$H_2PtCl_6 + 2K^+ \rightarrow K_2PtCl_6 \downarrow \text{ (yellow)} + 2H^+$$

$$K_2PtCl_6 + 6KI \rightarrow K_2PtI_6 \text{ (red-brown)} + 6KCl$$

$$K_2PtI_6 + 2Na_2S_2O_3 \rightarrow K_2PtI_4 \text{ (yellow)} + Na_2S_4O_6 + 2NaI$$

SPECIAL APPARATUS AND REAGENTS

1. *Rehberg Microburet (0.25 ml.).*

2. *Sulfuric Acid.* Concentrated H_2SO_4 (c.p.).

3. *Chloroplatinic Acid.* ($H_2PtCl_6 \cdot 6H_2O$) (10 ml. = 1 gm. Pt.). This is prepared by dissolving 26.57 gm. of $H_2PtCl_6 \cdot 6\,H_2O$ in 100 ml. of H_2O or by dissolving 17.27 gm. of $PtCl_4$ in 100 ml. of 1 N HCl.

4. *Ethyl Alcohol.* Absolute.

5. *Potassium Iodide 2 N.* This is prepared freshly for use by dissolving 8.3 gm. of KI in 25 ml. of distilled H_2O.

6. *Sodium Thiosulfate 0.1 N.* This is prepared by dissolving 49.6 grams of crystalline highest purity $Na_2S_2O_3 \cdot 5H_2O$ in recently-boiled, distilled water in a 2-L. volumetric flask, adding approximately 1.6 gram of NaOH (pellets) and diluting to the mark. The sodium thiosulfate solution is standardized with acid potassium iodate using starch solution as an indicator.

7. *Acid Potassium Iodate 0.1 N.* This is prepared by dissolving 0.3249 gm. of $KH(IO_3)_2$(c.p.) in 50 ml. of water in a small beaker, heating if necessary. The contents are then cooled and transferred to a 100-ml. volumetric flask, and diluted to the mark with distilled water.

8. *Soluble Starch Solution, 1 Per cent.* This is prepared by dissolving 1 gm. of soluble starch in 10 ml. of boiling distilled water and diluting to 100 ml. with a saturated solution of NaCl.

STANDARDIZATION OF SODIUM THIOSULFATE

Twenty-five ml. of 0.1 N acid potassium iodate are transferred to a 125 ml. Erlenmeyer flask and 1 gm. of KI and 5 ml. of 6 N HCl are added. The solution is titrated immediately with 0.1 N thiosulfate. When the solution becomes pale yellow, 1 ml. of the starch solution is added, and the titration is continued to the loss of blue color. The normality of the standard thiosulfate solution is calculated according to the following formula:

$$\text{Normality } Na_2S_2O_3 = \frac{25 \text{ ml. x } 0.1 \text{ N } (KH(IO_3)_2)}{\text{ml. } Na_2S_2O_3 \text{ required}}$$

PROCEDURE

1. One-ml. samples of serum or standard solution are transferred to 15-ml. Vycor centrifuge tubes.

2. One drop of concentrated H_2SO_4 is added to each tube.

3. The samples are dried in an oven at 100 C. for twelve to sixteen hours.

4. The tubes are placed in a cold muffle furnace, and the temperature is raised to 550° to 600°C.

5. The samples are ashed for four hours and are then cooled to room temperature.

6. Two-tenths (0.2) ml. of chloroplatinic acid solution is added to each centrifuge tube, with an 0.2-ml. blowout pipet.

7. The contents are stirred for one minute with a fine pointed stirring rod, to complete precipitation of potassium chloroplatinate (K_2PtCl_6). The stirring rod is left in the tube.

8. Five ml. of absolute ethyl alcohol

are added to each tube using a serologic pipet.

9. The contents are mixed thoroughly.

10. The stirring rod is rinsed with 1 ml. of absolute alcohol, and retained for use in step 17.

11. The tubes are allowed to stand for twenty minutes and then centrifuged for ten minutes at 2000 r.p.m.

12. The 0.1 N sodium thiosulfate solution is standardized at this time. Directions for the standardization will be found under "Reagents."

13. After centrifugation (step 11), the supernatant fluid is aspirated through a fine capillary tube.

14. Steps 8 through 11 and step 13 are repeated.

15. The tubes are placed in a water-bath at 70°C.

16. The water-bath is brought to boiling, and maintained at 100°C. for ten minutes to volatilize the alcohol completely.

17. While the tubes remain in the water-bath, 5 ml. of distilled water are added to each tube using a serologic pipet. The contents are stirred with the same stirring rod previously used, and the rod is left in the tube for the remainder of the procedure.

18. When solution of the potassium chloroplatinate is complete, 1 ml. of 2 N potassium iodide solution is added. The tubes are allowed to remain in the hot water-bath for five minutes to insure complete formation of potassium iodoplatinate.

19. The tubes are cooled to room temperature by immersion in a beaker of tap water.

20. The samples are titrated with 0.1 N sodium thiosulfate solution using the Rehberg microburet. The end point is the complete disappearance of the red color of potassium iodoplatinate, leaving the lemon-yellow color of potassium iodoplatinite.

CALCULATIONS

Potassium concentration (mEq. per L.) = ml. $Na_2S_2O_3$ x normality of $Na_2S_2O_3$ x 1000.

Estimation of Serum Potassium Using Tetraphenylboron[6,7]

PRINCIPLE

Tetraphenylboron reacts with potassium to produce a turbid suspension of potassium tetraphenylboron. At pH 3.5, with trichloracetic acid as the protein precipitant, the relationship of optical density to potassium concentration most nearly approaches linearity. Absorption measurements are made at 420 mμ and the concentration of potassium is obtained by reference to a calibration curve.

REAGENTS

1. *Tetraphenylboron Solution.* Exactly 5 grams of sodium tetraphenylboron reagent* are dissolved in 50 ml. of distilled water, and transferred to a 100-ml. volumetric flask. Ten ml. of 0.1 N NaOH are added, and the solution is diluted to the mark. The solution should be virtually clear. Turbidity, if it devel-

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*The sodium tetraphenylboron reagent employed in this procedure was obtained from the K & K Laboratories, 29-46 Northern Boulevard, Long Island City 1, New York. The reagent should be stored in a desiccator. If the tetraphenylboron is allowed to become hydrated, it may be placed overnight in a vacuum desiccator filled with anhydrous calcium sulfate, or it may be recrystallized from methylene chloride and dried at 120°C.

ops, can usually be traced to contamination with potassium or ammonium salts. If stored in the refrigerator, the tetraphenylboron solution is stable for two weeks.

2. *Alkaline Ethylenediaminetetraacetic Acid (EDTA) Solution.* Seven and one-half (7.5) grams of ethylenediaminetetraacetic acid disodium salt are transferred to a 500-ml. volumetric flask. Forty-four ml. of 1.0 N NaOH are added, and the solution is diluted to the mark with distilled water. This solution is stable for at least two weeks at room temperature.

3. *Formaldehyde Solution.* Formalin (37 per cent formaldehyde) is diluted 1:1 with distilled water.

4. *Mixed Alkaline EDTA-formaldehyde Solution.* This solution is prepared fresh by mixing two parts of reagent 2 with one part of reagent 3.

5. *Gum Ghatti Solution.* Ten grams of gum ghatti tears are placed in a bag made of a double layer of gauze. The bag is suspended in a beaker containing 400 ml. of distilled water so that the upper surface of the water just covers the contents of the bag. After leaving it overnight, the bag is removed and discarded. The solution is mixed, allowed to stand for several hours and then decanted and, if necessary, filtered.

6. *Trichloracetic Acid Solution, 8 Per cent (w per v).* Eighty grams of trichloracetic acid reagent are dissolved in distilled water and diluted to 1-L. in a volumetric flask.

7. *Potassium Standard Stock Solution.* Into a 1-L. volumetric flask, 0.3728 gm. of fused potassium chloride is carefully transferred and diluted to the mark with distilled water. This solution contains 5 mEq. of potassium per L.

8. *Potassium Standard Working Solutions.* Zero-, 2-, 4-, 6-, and 8-ml. samples of the potassium standard stock solution are transferred to 50-ml. volumetric flasks. Twenty-five (25) ml. of the 8 per cent trichloracetic acid solution are added to each flask and the contents are diluted to the mark with distilled water. When employed as outlined in the procedure, these standards correspond to concentrations of serum potassium of 0,2,4,6, and 8 mEq. per L., respectively, and these standards are used in constructing the calibration curve.

PROCEDURE

1. Trichloracetic acid filtrates of serum are prepared by adding 5 ml. of 8 per cent trichloracetic acid solution to 1 ml. of serum in a 10-ml. volumetric flask or calibrated tube. The contents are diluted to the mark with distilled water. After standing for ten minutes, the contents are filtered through Whatman No. 40 filter paper.

2. Three ml. of the mixed alkaline EDTA-formaldehyde solution are transferred to each of the colorimeter cuvets.

3. One-ml. samples of the potassium working standards and 1 ml. of the trichloracetic acid filtrate of serum are transferred to their respective cuvets.

4. Three drops of gum ghatti are added to each cuvet, and the contents are mixed.

5. One ml. of tetraphenylboron solution is added rapidly to each cuvet by blowing vigorously with an Ostwald-Folin blow-out pipet. The contents are mixed by swirling and allowed to stand for fifteen minutes.

6. Optical density readings are made at 420 mμ within fifteen to thirty minutes after mixing.

CALCULATION

A calibration chart is constructed by plotting the values for optical density as ordinate, and the concentration of potassium as abscissa. The concentration of potassium in the unknown serum is obtained by reference to the standard curve. Analyses yielding potassium concentrations greater than 8.0 mEq. per L. are repeated, using 0.5 ml. of the trichloracetic acid filtrate, and 0.5 ml. of blank solution.

MICRO-MODIFICATION

The following procedure may be employed when only small quantities of serum are available for analysis. One milliliter of trichloracetic acid reagent is transferred to a volumetric tube graduated at 2.0 ml.* A pipet calibrated to contain 0.2 ml. is used to expel serum into the trichloracetic acid precipitant. The contents of the tube are diluted to the 2 ml. mark with distilled water, mixed, and allowed to stand for ten minutes. After centrifugation, 1 ml. of the supernatant protein-free fluid is withdrawn for analysis, as described in the regular procedure (step 3).

DISCUSSION

Comparison of Methods

Duplicate analyses with the tetraphenylboron method were made on serums collected from thirty-three hospitalized patients. The results of these analyses were compared with those obtained by the platinic chloride method. The means of analyses by the two methods were identical, and the maximal deviation between the results of the two methods was ± 0.6 mEq. per L.

Estimation of Potassium in Serum

For clinical purposes, the estimation of potassium should be made on serum and not whole blood. The concentration of potassium in erythrocytes is about twenty times that of serum and may vary over a wide range, depending on the cellular volume. Care should be taken to avoid hemolysis of the serum sample.

The normal range of concentration in human blood serum is between 3.1 and 5.3 mEq. per L. with an average of 4.4.[5] The concentration of potassium in serum gradually increases on standing in contact with red cells. It is preferable to collect venous blood without stasis under oil. Separation of cells should be carried out as soon as possible to minimize error due to diffusion of potassium from cells to the surrounding fluid.

Factors which tend to increase serum potassium[3] are (a) increased potassium intake, (b) oliguria or anuria, (c) tissue breakdown, (d) anoxia, (e) contraction of extracellular volume by dehydration, and (f) adrenal cortical insufficiency. Factors tending to decrease serum potassium concentration are (a) limitation of food intake, (b) increased renal excretion of potassium, (c) diuresis, (d) acidosis, (e) adrenal cortical hyperactivity, (f) dilution of extracellular fluids, (g) vomiting or diarrhea, and (h) glucose uptake by cells. The concentration of potassium in serum at any given time represents a balance of many factors.

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*Arthur H. Thomas Co., 2 ml. volumetric tubes, No. 5474, are satisfactory.

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The Chemical Measurement of Calcium in Serum

ROBERT P. MacFATE, Ph.D.

A. General Considerations

Procedures for the measurement of calcium in biologic materials were reviewed in a previous volume of this series, *Evaluations of Thyroid and Parathyroid Functions*[42]. The basic procedures are listed in Table I. The literature of the past five years is here reviewed with special attention to the measurement of the concentration of calcium in serum. Many methods have been studied, tested, and modified during this period.

CHELATION METHODS

Van Schouwenburg[62] found interference of magnesium in the *EDTA* titration of calcium using *murexide* (ammonium purpurate). Using Carbocel as a protective colloid, the formation of magnesium hydroxide was prevented, and the interference was removed. This procedure was recommended especially where specimens contain a high concentration of magnesium and only a low concentration of calcium.

Copp[15] reported a fast, precise and semiautomatic micromethod for calcium in serum and urine which was essentially a photometric titration with *EDTA*, using *murexide* as the indicator. High precision was claimed for analysts who had very little laboratory experience. Compared with the Clark-Collip[13] method, plasma values were lower by 3 to 4 per cent and recovery of added calcium was 99 per cent.

Spare[58] has recently published an article on the effects of various solvents on the fading rate of alkaline *murexide*. A solution in propylene glycol, with the amount of water being limited, was stable for one week at room temperature and for three to four weeks if refrigerated.

Kingsley and Robnett[32] further investigated Baar's method[4] using *nuclear fast red* for the direct spectrophotometric determination of calcium in serum, urine and cerebrospinal fluid. The authors noted that the dye must be purified. The unreacted dye was used as a blank, and the decreasing densities in the presence of the unknowns were taken as a measure of the amount of calcium present. This procedure places a maximum "load" on the electronic system of the photometer, which may thus give nonlinear results if the photometer is not operating efficiently. Analytical results were in good agreement with established methods.

Table I Basic Procedures for the Determination of Calcium in Biological Materials

1. *Biologic Methods,* such as the isolated heart of the frog.
2. *Gravimetric Methods,* where calcium oxide and calcium picrolinate are weighed as the end-products.
3. *Volumetric Methods,* including
    (a) Acidimetry, using a standard acid solution.
    (b) Oxidimetry, using potassium permanganate or ceric sulfate.
    (c) Iodimetry, where the liberated iodine is titrated with thiosulfate.
    (d) Iodometry, where the excess oxidizing agent is back-titrated.
    (e) Chelation, titrating with *EDTA, EGTA,* or *DCTA,* with an indicator, or using the dye alone.
4. *Gasometric Method,* using the Van Slyke apparatus.
5. *Photometric Methods,* including the use of the spectrophotometer and the flame photometer.
6. *Nephelometric Methods,* such as the calcium oleate procedure and other turbidimetric methods.
7. *Spectrographic Method.*
8. *Polarographic Method.*
9. *X-ray Spectroscopy.*
10. *AutoAnalyzer Technics.*

McAllister and Yarbro[46] adapted the method of Yarbro and Golby[75] to the determination of calcium in serum. The procedure used *EGTA* (ethylene glycol-bis-(β-aminoethyl ether)-N, N′-tetraacetic acid) with *Calcon* as the indicator. *EGTA* was considered by the authors to be superior to *EDTA,* since it is a specific complexon for calcium in the presence of magnesium. Variations on replicate samples were within ± 4 per cent, and recoveries ranged from 94 to 103 per cent.

Bowden and Patston[9] adapted the method of Golby, Hildebrand and Reilley[20] to a commercial photoelectric titrimeter, due to the difficulty in determining the end-point by the original visual technic. *EDTA* was used with the dye *Calcon.* The interference of magnesium and phosphate was overcome. Results were slightly lower (−0.15 mg. per 100 ml.) than those obtained with the oxalate-permanganate method. Magnesium was estimated by incorporating the use of *Eriochrome black T.*

Malmstadt and Hadjiioannou[44] improved their original method[43] which automatically titrated the calcium, also using *EDTA* and the dye *Calcon.* Here, again, *Eriochrome black T* was used to estimate the magnesium concentration. Analyses were made within 10 seconds with a reproductibility of 1 to 2 per cent. Recoveries varied from 97 to 103 per cent.

Pappenhagen and Jackson[50] have suggested modifications for the micro method of Bachra, Dauer and Sobel[5] for determining serum calcium, using *EDTA* and *Cal-red* as suggested by Patton and Reeder[52]. The modifications increased the reliability of the standard curve, the stability of the end-point, the stability of the indicator, and the ease of manipulation. Reproducibility was reported to be within 0.1 mg. of calcium per 100 ml.

Using the method of Pappenhagen and Jackson[50], Burdick, Wildasin and Chang[12] reported that the normal range of serum calcium, by this method (*EDTA* and *Cal-red*), was from 8.2 to 10.3 mg. per 100 ml.

Due to the difficulty encountered in determining the end-point of calcium indicators in highly-pigmented (icteric) specimens, and feeling that spectrophotometric determination of the endpoint was more cumbersome than titration, Fingerhut and Miller[18] suggested that the major portion of the serum chromogens could be oxidized to colorless products, either by a mixture of hydrogen peroxide and potassium ferricyanide or by sodium hypochlorite. The decolorized solutions were titrated with *EDTA*, using either *Calcein* or *Cal-red*. Findings using *Calcein* were 0.25 mg. of calcium per 100 ml. of serum lower than the Clark-Collip[13] procedure, with a standard deviation of ±0.38 mg. per 100 ml. Results obtained using *Cal-red* were only 0.09 mg. per 100 ml. lower than the Clark-Collip[13] values, with a standard deviation of ±0.17 mg. per 100 ml.

Jackson, Breen and Chen[24] adapted the method of Bett and Fraser[8] to the fluorometer, using *EDTA* and *Calcein W*. The decrease in fluorescence, which occurs when *EDTA* removes calcium from the calcium-calcein complex, was measured during the direct titration of serum or urine. Recovery of calcium was complete, and good agreement was obtained with the Clark-Collip[13] procedure.

Kovács and Tárnoky[33] used the dye *Plasmocorinth B**, as suggested by Yanagisawa[73, 74] and described by Kingsley and Robnett[30, 31], to titrate both calcium and magnesium in the same sample of serum.

Brush[11] conducted a comprehensive study of the Kingsley-Robnett[30, 31] method, using *Erio SE* (identical with *Plasmocorinth B* and *Eriochrome Blue SE.*) He proposed an ultramicro method using the spectrophotometer, and he gave additional procedural details that simplified and improved the accuracy of the previous methods. He reported that closer temperature control must be exercised, and he confirmed the previous findings that lipemic sera must be extracted with ethyl ether, or false low values would be obtained. Conclusions are drawn as to the nature of the calcium ion-dye complex.

Radin and Gramza[53, 54] described a differential spectrophotometric technic for the determination of micro amounts of calcium, using *Eriochrome Blue SE*. They stated that the method shows greater sensitivity and accuracy than did previous methods using this dye. Study of various kits for the determination of micro amounts of calcium proved them to be inadequate for an accurate or precise calcium analysis.

Jones and McGuckin[27] suggested

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*Footnote**. There has been some confusion concerning the name and formula of this dye. Kovács and Tárnoky (33) called it *Plasmocorinth B*, supplied by Inabata Industry and Co., Ltd., Osaka, Japan. They referred to Yanagisawa (74) who obtained it from the same company, and to Kingsley and Robnett[30] who noted that it could be obtained, also, from Clinton Laboratories, Los Angeles. In an addendum to their paper, Kovács and Tárnoky (33) stated that the dye *Eriochrome Blue SE* supplied by Geigy A. G., Basel, Switzerland, is chemically identical with *Plasmocorinth B*.

Brush (11) stated that the dye *Eriochrome Blue SE*, produced by Geigy, was most commonly known as *Erio SE*. He also stated that this was the dye used by Yanagisawa (74) and Kingsley and Robnett (30, 31).

Radin and Gramza (53, 54) obtained the dye from Eastman and called it *Eriochrome Blue SE*, as did Jones and McGuckin (27). In private communications, Geigy Pharmaceuticals, Yonkers, N.Y., and the Chemical Abstracts Service state that the dyes are chemically identical.

Various formulas have been given which added to the confusion. In a private communication, the Chemical Abstracts Service states that the dye is listed now as Colour Index (C.I.) Mordant Blue 13 and that the systematic name is the Na salt of 3-[(5-chloro-2-hydroxyphenyl) azo]-4,5- dihydroxy-2,7-naphthalenedisulfonic acid.

a semi-automatic procedure for the direct titration of calcium and magnesium, also using *Eriochrome Blue SE*. The end-point is recorded automatically with respect to time. The results averaged 0.3 mg. of calcium per 100 ml. of serum less than those obtained by the Clark-Collip[13] procedure.

Lindstrom and Diehl[38] suggested the use of a new indicator called *Calmagite* which has the same color change as Eriochrome black T, but the color change is said to be clearer and sharper, and aqueous solutions of the indicator are stable indefinitely. The dye may be substituted for the older indicator without change in the procedure. Titration with *EDTA* was conducted at pH 10. The compound may be used, also, to determine the concentration of magnesium in serum.

Jackson and Brown[25, 26] adapted *Calmagite* to the *EDTA* titration of both calcium and magnesium in the trichloroacetic acid filtrate of serum. Successive photometric readings of per cent transmittance were plotted against the volume of *EDTA* added. Two sharp "breaks" were obtained in the titration curve, the first corresponding to the amount of calcium and the second to the amount of magnesium. Normal adults gave calcium values varying from 4.6 to 5.4 mEq. per liter. Replicate determinations showed a standard error of ± 0.08 mEq. per liter.

Watson and Rogers[65], following the suggestion of Mori[19], titrated a trichloroacetic acid filtrate of serum with *DCTA*. (sodium salt of 1, 2 diaminocyclohexane-N-tetraacetic acid octahydrate), using *Calcein* as the indicator. It was noted that the procedure may be used successfully in cases of hemolyzed or bile-pigmented sera. The mean and the standard error of their results on twenty icteric infant sera were 8.55 ± 0.18 mg. per 100 ml., as compared with 8.90 ± 0.20 mg. per 100 ml. by the method of Clark-Collip[13], modified by Varley[64].

Coolidge[14] reported on a method for calcium in ashed urine and bone which can be adapted as a micro method to the analysis of serum. The findings showed that reliable results could be obtained in the presence of magnesium and phosphate by the addition of excess *EDTA* and back titration with 0.100 M calcium chloride, as suggested by Yalman *et al.*[72]. *Calcein* was used as the indicator.

AUTOANALYZER METHODS

The increased use of the AutoAnalyzer* and some dissatisfaction with the recommended technics for calcium have resulted in several papers attempting to devise methods through which simultaneous or individual determinations could be made of calcium and phosphorus, in both serum and urine, without preliminary treatment of the samples.

The recommended AutoAnalyzer method was derived from the colorimetric procedure of Williams and Moser[68] using *murexide* at an alkaline pH.

Levy *et al.*[37] made a series of suggestions to improve the original AutoAnalyzer method recommended for serum. However, this procedure cannot be used for the determination of calcium in unprocessed urine. For urine, the calcium must be precipitated as the oxalate, dissolved, and then analyzed.

Wieme and van Raepenbusch[67] adapted to the AutoAnalyzer the direct spectrophotometric method of Kingsley and Robnett[30, 31] which was used for the manual analysis of serum and urine. *Corinth Ca* was used as the indicator; the diffusion step was omitted. Excellent reproducibility and less contamination were reported. The authors stated that there was no need for continuous calibration. Accurate results were reported for the direct determination of calcium in urine.

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*Technicon Instruments, Corporation, Chauncey, N.Y.

Kessler and Wolfman[28, 29] described a new automated procedure for measuring calcium and phosphate in serum, urine or other biological fluids, simultaneously or individually, without preliminary treatment of the sample. The dye used was *cresolphthalein* complexon, introduced by Anderegg *et al.*[2] and Schwarzenbach[56]. Separation of calcium (and phosphorus) from interfering materials was achieved by dialysis under acid conditions. Magnesium, phosphate, citrate, and sulfate, did not introduce significant errors. Phosphorus was determined by a modification of the Fiske and Subbarow[19] procedure. Findings correlated well with those by manual methods.

FLAME PHOTOMETRY

The recommended procedure for the flame photometry of calcium is discussed in Chapter 15 of this volume. A number of papers have appeared during the past five years. Parker[51] reviewed the use of the flame photometer and recommended the use of standards that contained sodium and potassium in the concentrations usually found in serum. It was noted that dilution of the sample with distilled water having a cation concentration different from that of the water used in preparing the reagents was a major source of error. By the Macro-Coleman method, the recovery of added calcium was 100.4 per cent; by the Micro-Coleman method it was 98.7 per cent.

Fawcett and Wynn[17] suggested dilution of the sample in 10 mM. magnesium sulfate containing 2 mM. sodium chloride, giving final calcium concentrations in the range of 0.05 to 0.10 mM. In this diluent, galvanometer readings were proportional to calcium concentrations up to 0.4 mM. The magnesium sulfate prevented depression of the calcium emission by phosphate and sulfate, and the excess sodium chloride eliminated enhancement of calcium emission by the added sodium and potassium in the sample. Subtraction of background readings excluded direct interference, and results on plasma were consistently 2 per cent higher by this technic than by *EDTA* titration.

Thiers and Hviid[59] suggested the use of laboratory-built "automatic background-subtraction" photometer to cancel the interference of cations in the flame photometric determinations of calcium in serum or urine samples. A chelating agent, ammonium *EDTA*, was used to prevent the interference of anions. The precision of the method and its accuracy were expressed as a range of ± 0.1 mEq. per liter.

Brandstein, Castellano, and Mezzacapa[10] found in the use of the Coleman model 21 flame photometer that the interference phenomenon, resulting from the intensifying effect of sodium upon calcium luminosity, could be corrected by applying a correction factor of 0.018 mEq. per liter of calcium for each mEq. per liter of sodium differential between the standard and sample. The correction factor was subtracted from the calcium result, if the sodium in the sample was above the level in the standard. Conversely, the correction factor was added, if the sodium in the sample was below the level in the standard. They stated that potassium, magnesium, sulfate, chloride, phosphorus, and total protein did not interfere within the relatively wide limits tested in this instrument. The standard must have protein present, and this may be either a commercial preparation or human serum on which the actual concentrations of calcium and sodium have been determined.

Love[40] described a procedure in which the calcium is precipitated as the oxalate. The calcium oxalate is dissolved in perchloric acid and then measured by flame photometry.

Webb and Wildy[66] suggested the use of a spectrograph for the determination of calcium in tissues and other biologic materials. The specimens were ashed and dissolved in hydrochloric acid; a plasma-jet was used, operating at an electron temperature of 8,000 degrees K. The intensity of the calcium line at 3933 Angstrom units was measured by an integrating flame photometer, and aqueous solutions of calcium were used for calibration.

Anderson and Weinbren[3], in the performance of calcium balances using chromium sesquioxide as an inert fecal reference substance, recommended the use of the flame photometer for the determination of both chromium and calcium. The feces was ashed, then dissolved in perchloric acid, and this solution was used for both chromium and calcium determinations. The accuracy of the findings was said to equal that of the chemical methods usually employed.

N-HYDROXYNAPHTHALIMIDE METHOD

Of the many colorimetric methods for the determination of calcium in serum, (heparinized) plasma, urine and cerebrospinal fluid, the most promising has been the N-hydroxynaphthalimide (naphthalhydroxamic acid) procedure described by Trinder[60] and by Gran[21]. The reagent was first prepared by Beck[7] and, subsequently, by Yasuo[76]. Sherrick and de la Huerga[57] modified the procedure.

Calcium is precipitated by N-hydroxynaphthalimide over a wide range of alkalinity. The yellow to orange-red complex that is formed is highly insoluble. In Trinder's procedure, calcium is precipitated directly from the specimen by the addition of a slight excess of the reagent. After centrifugation, the clear supernatant is removed by decantation and drainage. The unwashed precipitate is dissolved in alkaline *EDTA* solution, as suggested by Amin[1]. Trinder[60] and Sherrick and de la Huerga[57] added acid ferric nitrate solution to intensify the orange-red color to an amber hue, the intensity of which is a measure of the amount of calcium present in the serum. Readings are made in the spectrophotometer. Trinder[60] suggests reading at 450 mμ, and Sherrick and de la Huerga[57] recommend reading at 400 mμ. Gran[21] did not use the ferric nitrate solution, and he thus obtained a yellow solution which is read at 338 mμ.

Trinder[60] reported identical findings for the mean of fifty specimens of serum, as compared with the Clark-Collip[13] method. On twenty urine specimens, he obtained a mean of 14.2 mg. per 100 ml., as compared with 13.99

mg. per 100 ml. by the Clark-Collip[13] method.

Sherrick and de la Huerga[57] reported that the mean error was 2 per cent with a standard deviation of ± 0.96, and that added calcium was recovered satisfactorily.

MISCELLANEOUS METHODS

Improvements and recommendations have been made concerning a variety of other technics for the determination of calcium.

Ritter, Spencer, and Samachson[55] tested the accuracy of the *Sulkowitch test*[5] as an indicator of hyper- and hypocalcemia and of hyper- and hypocalciuria. Some correlation was found between the classifications of the test and concentration of calcium in urine, but there was considerable scatter, and the degree of correlation was too poor for usefulness in clinical interpretations. The authors stated that the test should be replaced, whenever possible, by quantitative determinations of urinary and/or serum calcium.

Willis[71] devised a procedure for the determination of calcium and magnesium in urine by *atomic absorption spectroscopy*. This followed earlier papers[69, 70] in which the method was used in serum analysis. The urine was diluted with a solution of either lanthanum chloride or strontium chloride. Values obtained agreed well with those obtained by the oxalate-permanagante procedure.

Irving and Watts[23] developed a *polarographic method* for the estimation of calcium and magnesium in sera. In this method the total calcium and magnesium were determined first and then the magnesium alone. The calcium concentration was found by difference. The accuracy and reproducibility of the method compared favorably with those of the Clark-Collip[13] method, although the polarographic estimates tended to be slightly higher though not statistically significant. This may have been due to possible loss by dissolving of the precipitate in the Clark-Collip[13] procedure.

Van Slyke and Carson[63] suggested a simplification of the procedure for the determination of small amounts of calcium as the oxalate. The calcium oxalate precipitate was both formed and washed in a filter tube with a *fritted-glass filter*. The time required for manipulation was minimized, and the sources of error encountered in washing by centrifugation were avoided. The washed precipitate was redissovled in a solution of perchloric acid, and the dissolved oxalate was either titrated or determined manometrically.

Mathies and Lund[45] reported further studies on the use of *x-ray fluorescence or x-ray emission spectroscopy* for the determination of microgram quantities of calcium in blood serum.

The determination of protein-bound, ionized and diffusible calcium is considered in Chapter 5. It is of interest to note that three papers[16, 41, 61] appeared in the past five years making use of the metal indicator murexide. One paper[39] used the flame photometer, and another[22] used the Clark-Collip[13] procedure.

DISCUSSION

Ever since the introduction by McCrudden[47, 48] in 1909 of the oxalate-permanganate titration for the determination of calcium in biologic materials, this basic procedure has been used in the measurement of calcium. Notwithstanding its many good points, the oxalate-permanganate method for calcium in serum is not ideal as a *routine clinical laboratory procedure*. Extreme care must be exercised in the technic used, although this is no problem for an experienced chemist. The major objections are that 2 ml. of serum must be used to achieve the desired sensitivity, and the procedure requires at least two hours for its completion.

In the light of the present-day trend toward simplification of clinical laboratory methods, many shorter technics using much less serum (0.2 or 0.1 ml.) have been suggested as routine procedures. Each of these methods has its inherent errors with which the analyst must be completely familiar.

Whatever routine procedure is used, it should be checked periodically by a *reference method*. It is generally accepted that the Clark-Collip[13] modification of the Kramer-Tisdall[34–36] method is best suited for this purpose since, under diverse and exigent conditions, the stoichiometry of the reaction is invariable and, with proper attention to technic, consistent findings are obtained.

With detailed study, the *chelation technics* have not proved to be the great success first expected of them.

The various complexometric procedures are still plagued with nonspecificity. Some of the complexons will unite with calcium only; others under adjusted conditions, will also unite with magnesium. In many cases the absolute specificity of the reaction is questionable. Phosphates will interfere to a greater or lesser degree.

To overcome these problems, a variety of complexons and indicators have been tried. Some analysts believe *EGTA* (the ethylene glycol tetracetate) and *DCTA* (the diamino cyclohexane tetraacetate) to be superior to *EDTA* (the ethylene diamino tetraacetate), which is used most commonly.

Concerning the dyes or indicators, a great number have been used. These included *murexide, nuclear fast red, Calcon, Cal-red, Calcein, Calmagite, Plasmocorinth B* (also called *Eriochrome Blue SE)*, and others. Problems have included the purity of the dye, the instability or fading of the dye solution, poor visual end-points, the stability of the end-points, obscuring of end-points by icteric specimens, and a marked dependance on the pH.

In some of the technics, it has been noted that the chosen end-point varies not only with different analysts, but from day to day with the same analyst. Some of the newer indicators appear to give more definite end-points.

Greater accuracy has been obtained where a direct reading is made with the spectrophotometer or fluorometer, or where the titration curve is charted. If a chelation procedure is to be used, this technic of reading is recommended. In such methods, the analyst requires less over-all experience for the performance of the test. It is to be noted that the highest quality of instrument will give the best results.

To overcome interfering factors, a variety of modifications have been tried, such as the preparation of trichloroacetic acid filtrates and the ashing of the sample with precipitation of the calcium as the oxalate. Technics have been used to bleach the icteric color from some specimens, and back-titrations have been tried.

No one chelation procedure has proved to be superior to all others. If such a technic is chosen, the analyst should be thoroughly familiar with the inherent errors of the method, the necessity for absolute adherence to the recommended technic, and the possibly wider range of normal findings than are obtained by some other procedures. Comercially prepared *kits* for the determination of calcium are not recommended.

The *AutoAnalyzer procedure* appears to give satisfactory findings at the expense of some complications. Extreme care must be taken to note when contamination may occur. Standards must be repeatedly tested with a series of unknowns.

It must be noted in diffusion methods that the diffusion of calcium from a solution containing protein differs from that of diffusion from an aqueous solution, which is used as the standard in some methods. Accordingly, methods have been proposed omitting the diffusion. Generally, this has markedly lowered contamination.

Methods difficult to control manually appear, in many cases, to be adaptable to the standardized conditions found in the AutoAnalyzer. A variety of indicators have been used. Many of the methods for serum cannot be used for untreated urine. In these procedures the urinary calcium is precipitated as the oxalate, isolated, and dissolved, and the solution is used for analysis. This, of course, introduces additional steps and complicates the procedure.

Most of the suggested methods have been aimed at the direct analysis of unprocessed serum and urine. Further reports will be necessary before conclusions can be made about these newer methods.

The flame photometric technic is discussed in detail in Chapter 15. Newer procedures have been aimed at removing the interference of protein, anions, and other cations. Removal of protein and ashing do not remove the interference of anions and other cations. A number of modifications have been suggested, such as various means of increasing the flame temperature. These have appeared helpful, and accuracy has been increased when background interference has been automatically corrected. The most attractive features of the flame photometric technic are its inherent simplicity and speed of the analysis.

Of the many colorimetric procedures, *the hydroxynaphthalimide method* has proved to be the most promising. The calcium is precipitated directly from the specimen; the complex is quite insoluble; there is little need to wash the precipitate; the calcium is precipitated over a wide range of alkalinity; and the method can be scaled down to micro quantities. Use of the procedure has given excellent results.

A variety of *miscellaneous technics* have been proposed. Many of these require special, and often expensive, equipment. It may well be that, in the future, x-ray emission spectroscopy and atomic absorption spectroscopy may replace our present procedures.

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B. Oxalate-permanganate Method for the Measurement of Calcium in Serum

REFERENCE METHOD

(Clark-Collip Modification of the Kramer-Tisdall Method)[1–4]

PRINCIPLE

Calcium is precipitated directly from the serum as the oxalate, which is washed, dissolved in sulfuric acid and titrated with potassium permanganate.

The following equations represent the chemical reactions involved:

$$CaCl_2 + (NH_4)_2C_2O_4 \rightarrow CaC_2O_4 \downarrow + 2NH_4Cl$$
$$CaC_2O_4 + H_2SO_4 \rightarrow CaSO_4 + H_2C_2O_4$$
$$5H_2C_2O_4 + 2KMnO_4 + 3H_2SO_4 \rightarrow K_2SO_4 + 2MnSO_4 + 8H_2O + 10CO_2$$

REAGENTS

All water used must be distilled and then run through a demineralizer.

1. *Ammonium oxalate,* 4 per cent aqueous solution (w/v)

2. *Ammonium hydroxide,* 2 per cent aqueous solution (v/v)

3. *Sulfuric acid,* approximately 1 N solution. Concentrated reagent-grade sulfuric acid, in the amount of 28 ml.,

is dissolved and diluted to 1 liter with demineralized distilled water.

4. *Potassium permanganate,* stock solution, approximately 0.1 N. Reagent-grade potassium permanganate, in the amount of 3.15 gm., is dissolved in approximately 900 ml. of demineralized distilled water. The solution is allowed to stand for about one week. It is then filtered through glass wool, diluted to approximately 1 liter with demineralized distilled water, and stored in a brown glass bottle.

5. *Potassium permanganate,* approximately 0.01 N solution. The stock permanganate solution (Reagent 4), in the amount of 100 ml., is diluted to approximately 1 liter with demineralized distilled water and stored in a brown glass bottle. The strength of this solution may change on standing and must therefore be determined each time the solution is used.

6. *Sodium oxalate,* 0.01 N solution. Reagent-grade sodium oxalate, which has been dried at 100° C. for at least twelve hours, in the amount of 0.6700 gm. is dissolved in approximately 200 ml. of demineralized distilled water and transferred quantitatively to a 1-liter volumetric flask, using demineralized distilled water to make the transfer. Five ml. of concentrated reagent-grade sulfuric acid are added and the solution diluted to 1 liter, exactly, with demineralized distilled water. The solution is mixed and preserved with a few drops of toluol. (*Note*:A 0.1 N solution may be prepared and diluted 1 to 10 to make a 0.01 N solution, if desired.)

SPECIAL APPARATUS

1. *Centrifuge tubes,* 15 ml., borosilicate glass, with fine tip. (Tubes with a capillary tip may be obtained from Rascher and Betzold, Inc., 730 North Franklin St., Chicago, Illinois, 60610. With these tubes a stirring rod is necessary, prepared by inserting a platinum or nichrome wire in a piece of very fine capillary tubing and fusing the two together.)

2. *Micro-buret,* 5 or 10 ml., graduated in 0.01 or 0.02 ml.

PROCEDURE

Specimens of blood, as required, are collected, allowed to clot, and the sera separated within one-half hour of collection. Analysis may be delayed twenty-four hours at room temperature if the serum is sterile.

The required number of centrifuge tubes are cleaned in acid, washed, dried and appropriately marked (it is not necessary that they be dry). Two ml. of water followed by 2 ml. of blood serum are added to each tube and mixed (a Vortex mixer may be used). One ml. of ammonium oxalate solution is added to each tube and thoroughly mixed. The tubes are stoppered and permitted to stand for at least one hour. They may stand over-night.

The tubes are centrifugalized at an RCF of about 1,000 (3,000 r.p.m. with a rotating radius of at least six inches) for five minutes until the precipitate is well packed in the tip (an angle-head centrifuge is not satisfactory). The supernatant fluid is decanted from each by an inversion of the tube in one quick movement over a waste jar or beaker. The tube is kept in the vertical position and while still inverted is placed on a a piece of filter paper or in a rack over filter paper or gauze and allowed to drain for one minute. The excess liquid is wiped from the mouth of the tube with absorbent paper and the tube carefully examined to determine that no calcium oxalate has been lost. If this occurs, a thin trail of fine granules may be seen down the side of the tube and the test must be repeated.

About 3 ml. of 2 per cent ammonium hydroxide solution are then added, preferably from a wash bottle with a fine tip, washing down the sides of the tube as the addition is made. The tube is tapped until the precipitate is broken apart and well mixed (a vortex mixer may be used). The tube is centrifugalized for five minutes and decanted as before. The precipitate is washed twice more (three times in all).

After the last decantation, 2 ml. of N sulfuric acid are added, and the tube is placed in a water-bath at 70 to 75° C. (A liter beaker of water may be used as a bath. A buret may be supported over the beaker, which in turn rests on a small (6 inch) electric heater controlled by a simple thermostat set to hold the water at 70 to 75° C. The tube may be supported in the bath by using two wire test-tube holders with the handles extended in opposite directions and the jaw of one clamp closed over the other around the tube.) The solution is stirred with a thin glass rod or with the special stirring rod described under "Special Apparatus," until the oxalate is completely dissolved. It is then titrated at 70 to 75° C. with the approximately 0.01 N potassium permanganate from a micro-buret. The end-point is a faint pink color that persists for fifteen seconds.

A blank, consisting of 2 ml. of N sulfuric acid and 1 ml. of distilled water, is titrated at 70 to 75° C. to the same end-point.

A titration to determine the strength of the permanganate solution is performed by adding 1 ml. of the 0.01 N sodium oxalate solution to each of three tubes. Two ml. of N sulfuric acid are added to each and the mixture is titrated at 70 to 75° C. to the same end-point. The amounts of permanganate used in the three titrations are averaged.

CALCULATION

(a) *Determination of the strength of the potassium permanganate solution:*

$\frac{1}{K-B} \times 0.2 = F$, the number of mg. of calcium equivalent to 1 ml. of the permanganate solution used,

where 1 = number of ml. of 0.01 N sodium oxalate used,
K = number of ml. of permanganate solution used to titrate the sodium oxalate, and
B = number of ml. of permanganate solution used to titrate the blank.

(b) *Determination of concentration of calcium in the unknown:*

$(P-B) \times F \times 50 =$ mg. of calcium per 100 ml. of serum,

where P = number of ml. of permanganate solution used to titrate the unknown sample,
B = number of ml. of permanganate solution used to titrate the blank, and
F = the factor determined previously.

$$\frac{\text{Mg. of calcium per 100 ml.} \times 20}{40.08}$$

= mEq. of calcium per liter.

NORMAL VALUES

Normal blood serum contains from 9.0 to 11.0 mg. of total calcium per 100 ml., or 4.5 to 5.5 mEq. per liter.

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C. N-Hydroxynaphthalimide Method for the Estimation of Calcium in Serum

The *colorimetric method* using N-hydroxynaphthalimide is recommended for routine determinations of calcium in bilogical fluids.

Beck[1] and Yasuo[4] described the preparation of the N-hydroxynaphthalimide reagent. Sherrick and de la Huerga[2] adapted the method for routine use. The Trinder[3] adaptation to the micro determination of serum calcium follows.

PRINCIPLE

Calcium is precipitated directly from serum by the addition of a slight excess of the N-hydroxynapthalimide. The orange-red complex formed is highly insoluble. After centrifugation, the clear supernatant is removed by decantation and drainage. The unwashed precipitate is dissolved in alkaline ethylenediaminetetraacetate solution. Addition of acid ferric nitrate intensifies the orange-red color to an amber hue, the intensity of which is a measure of the amount of calcium present in the serum. The equations in Figure 1 represent the chemical reactions involved.

REAGENTS

(All water used must be distilled and then run through a demineralizer.)

1. *Calcium Reagent.* A 250-ml. beaker is marked with a marking pencil to indicate a volume of approximately 100 ml. Two hundred fifty mg. of reagent-grade N-hydroxynaphthalimide (naphthalhydroxamic acid) are transferred to the beaker with the aid of a small amount of demineralized distilled water. Two grams of reagent-grade tartaric acid are transferred to the same beaker with the aid of a little water; 5 ml. of reagent-grade 2-aminoethanol (ethanolamine) are added; and the mixture is diluted to approximately 100 ml. The mixture is heated on an electric hot plate at moderate heat, stirring with a glass rod, using a hot plate with magnetic stirring, until solution is complete. The solution is permitted to cool spontaneously to room temperature (it

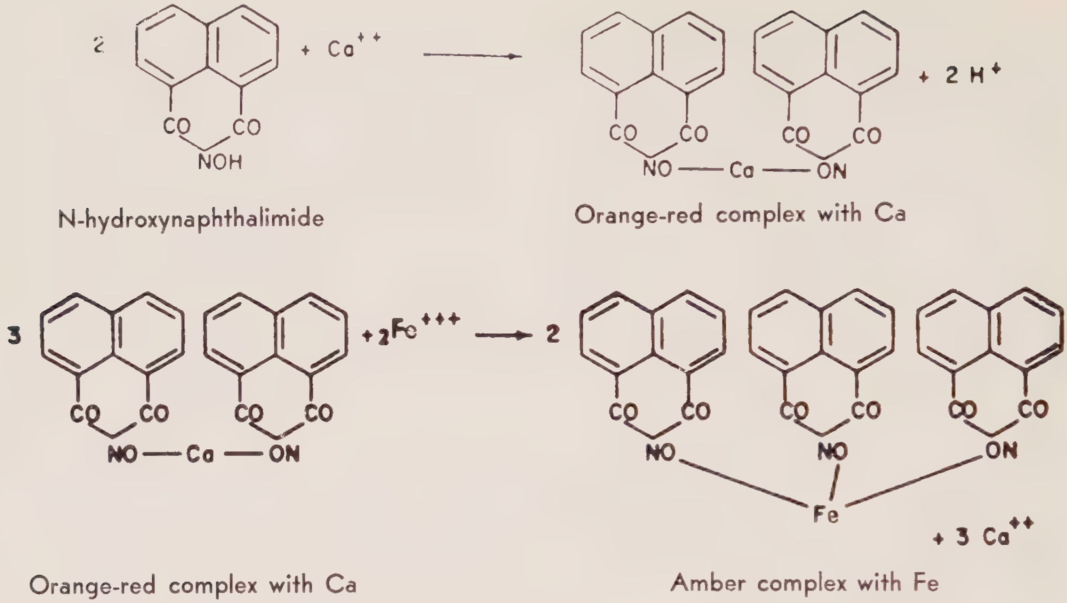

Fig. I

should *not* be chilled by immersing in cold tap water).

The prepared solution is transferred quantitatively to a 1-liter volumetric flask and a solution of 9 gm. of reagent-grade sodium chloride in approximately 500 ml. of demineralized distilled water is added. The mixture is then diluted to the 1-liter mark with demineralized distilled water and mixed thoroughly. The reagent is filtered through Whatman No. 42 filter paper, or the equivalent, and stored in a borosilicate or polyethylene bottle. The glass bottle is preferred so that one may visualize any cloudiness which may develop. If cloudiness develops, the reagent is again filtered through Whatman No. 42 filter paper.

The reagent is stable for at least six months at room temperature and may keep indefinitely.

2. *Alkaline EDTA Solution.* Two grams of reagent-grade disodium ethylenediaminetetraacetate (dihydrate) are transferred to a 1-liter volumetric flask and 10 ml. of 10 N sodium hydroxide added. The mixture is diluted to the 1-liter mark with demineralized distilled water and mixed thoroughly until all of the solute is completely dissolved. The solution is transferred to a polyethylene bottle and kept tightly stoppered.

The solution is stable for at least six months at room temperature and probably will keep indefinitely.

3. *Color Reagent.* Sixty grams of reagent-grade ferric nitrate (9 H_2O) are weighed-out in a tared 100-ml. beaker and transferred to a 1-liter volumetric flask with the aid of demineralized distilled water. Water is added to a volume of about 500 ml. and the mixture thoroughly shaken until all of the solute is completely dissolved. Fifteen ml. of reagent-grade concentrated nitric acid are added, the mixture diluted to the 1-liter mark with demineralized distilled water, and mixed thoroughly. The reagent is filtered through Whatman

No. 42 filter paper, or the equivalent, and stored in a borosilicate or polyethylene bottle. The glass bottle is preferred so that one may visualize any particulate matter which may develop. If particulate matter develops, the reagent is again filtered through Whatman No. 42 filter paper.

The reagent is stable for at least six months at room temperature and may keep indefinitely.

4. *Stock Standard Calcium Solution.* Primary standard calcium carbonate is dried at 110 to 120° C for three hours and cooled to room temperature in a desiccator. A sample of this material, in the amount of 0.4995 gm., is weighed-out and transferred to a 1-liter volumetric flask with the aid of approximately 200 ml. of demineralized distilled water. To the flask is carefully added 1.25 ml. of reagent-grade concentrated hydrochloric acid and the mixture gently shaken laterally until all of the solute is dissolved. The solution is diluted to the 1-liter mark with demineralized distilled water and mixed thoroughly. It is then stored in a glass-stoppered, or poly-seal cap-stoppered, borosilicate bottle and kept tightly stoppered. If mold develops, the solution is discarded.

The solution is stable for at least four years at room temperature, if a suitable mold-inhibitor is added.

One ml. of this solution contains 0.2 mg. of calcium.

SPECIAL APPARATUS

1. *Centrifuge tubes,* 12 ml., borosilicate glass, heavy duty. Preferably, special tubes with a capillary tip are used. These are described under "Special Apparatus" for the reference method (Section B of this Chapter).

2. *Cuvets,* 10 mm. (O.D.), Coleman.

CALIBRATION CURVE

Calcium working standard solutions are prepared from the stock standard solution as follows:

A solution equivalent to 10 mg. of calcium per 100 ml. is prepared by transferring 5.00 ml. of the stock standard solution to a 25-ml. Erlenmeyer flask and adding 5.00 ml. of demineralized distilled water. The flask is stoppered tightly and mixed thoroughly.

A solution equal to 5 mg. of calcium per 100 ml. is prepared by transferring 5.00 ml. of the previously prepared 10 mg. solution to a 25-ml. Erlenmeyer flask and adding 5.00 ml. of demineralized distilled water.

The centrifuge tubes to be used are labeled "B" for the blank, and 5, 10, 20 and 30 for the standards containing that number of mg. of calcium per 100 ml.

To each tube is added 2.5 ml. of calcium reagent. The 5, 10 and 20 mg. standards receive 0.10 ml. of the corresponding standard calcium solution. The 30 mg. standard received 0.15 ml. of the 20 mg. standard calcium solution.

The contents of the tubes containing the calcium standard are mixed well by vigorous tapping or on a Vortex mixer.

Each tube is then placed in a liquid bath at 100° C. for ten minutes or allowed to stand for fifteen minutes at room temperature and carried through the analysis as given under "Procedure."

The measurements, in terms of per cent transmittance, are plotted on semi-log paper against the mg. of calcium per 100 ml. The graph will be a straight line up to about 17 mg. per 100 ml. and will then curve slightly upward to 30 mg. per 100 ml. The graph may be extrapolated to 10.0 per cent transmittance.

PROCEDURE

The centrifuge tubes to be used are labeled "B" for the blank and an appropriate identification for each unknown.

To each tube are added 2.5 ml. of the calcium reagent. To the unknown tubes, 0.10 ml. of serum are added, using a "to contain" pipet. The pipet is rinsed three times with the reagent in the tube. The contents of the tubes containing serum are mixed well by vigorous tapping or on a Vortex mixer.

All of the tubes may then be placed in a liquid bath at 100° C. for ten minutes and cooled in cold tap water for one minute, or the tubes may be allowed to stand for fifteen minutes at room temperature (22° to 28° C.). During this treatment, the calcium will be completely precipitated as calcium naphthalhydroxamate.

All of the tubes are centrifugalized at an RCF of about 1,000 (3,000 r.p.m. with a horizontal radius of rotation of at least six inches) for at least three minutes.

The clear supernatant fluid is decanted from each tube by an inversion of the tube in one quick movement over a waste jar or beaker. The tube is kept in a vertical position, and while still inverted, is placed on a piece of filter paper or in a rack over filter paper or gauze and allowed to drain for at least three minutes. The inside of the mouth of each tube is wiped with gauze or absorbent tissue, and any droplets on the side are removed with a cotton-tipped applicator stick, being careful not to touch any of the precipitate.

To each tube is added 0.50 ml. of the alkaline *EDTA* solution, and the contents are mixed well by vigorous tapping, or on a Vortex mixer, until all of the precipitate is dispersed homogeneously. The tubes are then stoppered tightly with rubber stoppers, or preferably with rubber centrifuge tube caps, and placed in a 100° C. liquid bath for at least three minutes. Occasionally the tubes are shaken and are removed when all of the precipitate is completely dissolved to form a yellow to brick-red solution, depending on the concentration of calcium present. The tubes are cooled in cold tap water for one minute.

To each tube is added 1.50 ml. of color reagent; each tube is stoppered, and the contents mixed well by at least three inversions. The tubes are placed in a rack, and the time is noted, or a timer may be started. All of the solution is then transferred from each tube to properly labeled 10-mm. (O.D.) cuvets.

No less than twelve minutes and no longer than twenty-four minutes after adding the color reagent, the transmittance of each unknown is measured in the spectrophotometer at a wavelength of 450 mμ, setting the blank at 100 per cent transmittance.

If the per cent transmittance is less than 10.0 and the solution is within the period of color stability, twelve to twenty-four minutes after the addition of the color reagent, 1.00 ml. of the blank and 1.00 ml. of the sample may be transferred to properly labeled, clean 10-mm cuvets. To each cuvet is added 0.25 ml. of alkaline *EDTA* and 0.75 ml. of color reagent. Each cuvet is mixed well by inversion, the transmittance again measured, and the results from the calibration chart multiplied by two.

If beyond the twenty-four minute period, the entire test is repeated using only 0.05 ml. of serum. The final results are multiplied by two.

CALCULATION

The concentration of calcium is read in terms of mg. per 100 ml., directly from the calibration chart. Mg. per 100 ml. divided by two equals mEq. per liter of calcium.

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The Chemical Measurement of Magnesium in Biological Fluids

A. General Considerations

F. WILLIAM SUNDERMAN, JR., M.D. and F. WILLIAM SUNDERMAN, M.D., Ph.D.

Renewed interest in the changes in the concentrations of magnesium in pathologic conditions has stimulated the investigation of reliable methods for routine use. Magnesium methods, in general, may be classified into 7 categories: methods employing (1) the precipitation of magnesium as magnesium ammonium phosphate; (2) precipitation of magnesium as magnesium 8-hydroxyquinoline; (3) color reaction of magnesium with titan yellow; (4) color reaction of magnesium with carboxanilide dyes (5) *EDTA* titration using a variety of compleximetric indicators; (6) flame photometry; and (7) special methods involving ion exchange, atomic absorption, emission spectrography, X-ray spectroscopy, and polarography.

Magnesium Ammonium Phosphate Methods. These methods for measuring magnesium in biological fluids involve the precipitation and removal of calcium as oxalate and subsequent precipitation and isolation of magnesium as magnesium ammonium phosphate ($MgNH_4PO \cdot 6H_2O$). In the early methods, the magnesium ammonium phosphate was ignited to form magnesium pyrophosphate ($Mg_2P_2O_7$) which was determined gravimetrically. Titrimetric measurements of magnesium ammonium phosphate were also employed[1]. However, most of the magnesium ammonium phosphate procedures in current use involve the colorimetric measurement of phosphorus either by reduction of phosphomolybdic acid to molybdenum blue[2, 3, 4] or by reaction of phosphorus with molybdivanadate[5, 6].

8-Hydroxyquinoline Methods. Precipitation of magnesium 8-hydroxyquinoline from hot ammoniacal solution may be followed by gravimetric[7], titrimetric[8], colorimetric[9], flame photometric[10] or fluorimetric[11, 52] estimations. Ethanolic solutions of magnesium 8-hydroxyquinoline exhibit fluorescence with greatest intensity at 530 $m\mu$ when activated by light at 420 $m\mu$. This property is used as the basis for the rapid and sensitive fluorimetric procedures for measurement of magnesium.[11, 52]

Titan Yellow Method. The titan yellow method, first devised by Kolthoff in 1927[12], is based upon the reaction of titan yellow with magnesium hydroxide at alkaline pH to form a red lake, the intensity of which is a function of the concentration of magnesium. A number of modifications of the original method have been devised[13, 14, 15, 16]. In the procedure of Andreason[14] polyvinyl alcohol is used to increase the sensitivity of the titan yellow reaction. In the procedure of Neill and Neely[13] gum ghatti is employed to stabilize the color. Anast[51] has reported that gluconates interfere with the measurement of magnesium by the titan yellow method. With this method, artefacticiously low results were found in the serum and urine of patients who received calcium gluconate. A micromethod for the estimation of magnesium in biological fluids employing titan yellow has recently been reported.[53]

Carboxanilide Dye Method. In 1956, Mann and Yoe[17] reported a new dye for the determination of magnesium which they found to be more specific than titan yellow, and eight times as sensitive. This reagent, sodium 1-azo-2-hydroxy-3 (2, 4-dimethyl-carboxanilido) naphthalene-1 (2-hyroxybenzene-4-sulfonate) has been used by Bohoun[18] for the determination of magnesium in biological fluids. This method is described in Section C of this Chapter. A rapid ultramicro adaptation of this procedure has recently been reported.[54]

Schwarzenbach (EDTA) Titration Procedures. Several variants of the Schwarzenbach titration have been introduced for the measurement of magnesium in biological fluids. In the procedures of Carr and Frank[19] and Levine and Cummings[20] calcium is precipitated as oxalate and the magnesium in the filtrate is titrated with *EDTA* using *Eriochrome black T* as the indicator. In the *EDTA* procedures of Zak *et al.*[21] and Wilkinson[22] *murexide* is used as the indicator for calcium and *Eriochrome black T* as the indicator for total divalent cations (Ca + Mg). Magnesium is measured as the difference between the *murexide* and *Eriochrome black T* end-points. Kóvacs and Tárnoky[23] describe a simple compleximetric titration of serum calcium and magnesium using *Plasmocorinth B* as an indicator. When *EDTA* is added to a solution of calcium and magnesium ions, in the presence of sodium hydroxide, *Plasmocorinth B* forms a blue-violet complex after calcium has been bound. On the other hand, in the presence of ammonium hydroxide, color change occurs with *Plasmocorinth B* after *EDTA* has bound both calcium and magnesium. Magnesium may thus be estimated by difference from these two end-points. A number of other *EDTA* procedures have also been described.[24–32, 55, 56]

Flame Photometric Methods. Flame photometric methods for the estimation of magnesium in serum are relatively difficult due to the low concentration of serum magnesium, the low intensity of its emission lines, and the depression of magnesium emission by various anions. Teloh's procedure[36] is a self-standardizing method that requires two samples, to one of which a known amount of magnesium has been added. The method of Fawcett and Wynn[40] involves the preparation of perchloric acid filtrate of serum and the use of strontium-EDTA as a diluent to minimize the depression of the magnesium emission by anions. Direct methods of flame photometry utilizing spectrophotometers of high resolution have been proposed by Alcock, MacIntyre and Radde[38] and Montgomery[37]. Several additional modifications of the flame photometry of magnesium may also be noted.[33-37]

Special Methods. The separation of calcium and magnesium in biological fluids using a cation exchange resin has been described by Stevenson and Wilson[41]. Other promising new methods include the use of atomic absorption spectrophotometry[42] and the cathode-ray polarography[43]. Methods for determination of magnesium by atomic absorption spectrophotometry and x-ray spectroscopy are described in other chapters of this book.

NORMAL VALUES FOR MAGNESIUM IN SERUM

INVESTIGATOR	MEAN (mEq./L.)	RANGE (mEq./L.)
Sunderman[44]	1.96	1.8-2.2
Hald[45]	1.70	1.4-2.4
Alcock *et al.* [38]	1.66	1.5-1.8

MAGNESIUM-BINDING PROPERTY OF THE SERUM PROTEINS

Serum magnesium may be partitioned into diffusible and non-diffusible moieties and the magnesium-binding power of the albumin and globulin fractions in serum may be estimated. According to the studies of Sunderman, Copeland and Willis,[46–48] the magnesium-binding power of serum albumin is 0.0128 ± 0.004 mEq. per gram of albumin and the magnesium-binding power of serum globulin is 0.0081 ± 0.003 mEq. per gram of globulin.

CLINICAL INTERPRETATION OF SERUM MAGNESIUM

For a concise review of serum magnesium in normal and pathologic conditions the reader is referred to Chapter 23, and to the article by Hanna[49]. Hanna failed to include the noteworthy relationship between hypothermia and hypermagnesemia[50].

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B. Magnesium Ammonium Phosphate Method for the Measurement of Magnesium

F. WILLIAM SUNDERMAN, M.D., Ph.D., and F. WILLIAM SUNDERMAN, JR., M.D.

The time-honored isolation of magnesium as magnesium ammonium phosphate[12] and the colorimetric estimation of phosphorus by the method of Fiske and SubbaRow[3] is recommended as the reference procedure for use in clinical laboratories.

PRINCIPLE

Magnesium is determined in the serum following precipitation of calcium as calcium oxalate. Magnesium is then precipitated as magnesium ammonium phosphate ($MgNH_4PO_4 \cdot 5\ H_2O$). This compound is treated with ammonium molybdate to form phosphomolybdic acid, which is then reduced to molybdenum blue by aminonaphtholsulfonic acid. The molybdenum blue is measured photometrically.

REAGENTS

1. *Ammonium Oxalate, 4 per cent.* Four grams of ammonium oxalate (reagent grade) are dissolved in distilled water and diluted to 100 ml.

2. *Ammonium Hydroxide, 2 per cent.* Two ml. of concentrated ammonium hydroxide, (sp. gr. 0.9) are diluted to 100 ml. with distilled water.

3. *Potassium Dihydrogen Phosphate.*

Two grams KH_2PO_4 (reagent grade) are dissolved in 100 ml. of distilled water. This should be freshly filtered before using.

4. *Concentrated Ammonium Hydroxide.* Reagent grade, sp. gr. of 0.9.

5. *Alcoholic Wash Solution.* This is prepared by adding 200 ml. of 95 per cent v/v ethyl alcohol and 50 ml. of concentrated ammonium hydroxide to a liter volumetric flask, which is then filled to the mark with distilled water and mixed thoroughly. This solution is kept in the refrigerator.

6. *Ammonium Molybdate Reagent, 0.0083 N.* Precisely 2.566 gm. of reagent grade ammonium molybdate are placed in a 250 ml. volumetric flask. About 175 ml. of distilled water and 35 ml. concentrated H_2SO_4 (sp. gr. 1.84) are added while swirling the flask. Then water is added to the level of the neck of the flask. After cooling, water is added to the mark and the contents are mixed.

7. *Aminonaphtholsulfonic Acid.* Thirty gm of sodium bisulfite ($NaHSO_3$) and 1 gm. of sodium sulfite ($Na_2SO_3 \cdot 7\,H_2O$) are dissolved in 200 ml. of water. One half gm. of 1-amino-2-naphthol-4-sulfonic acid is added and mixed thoroughly and placed in a dark glass bottle. This colorless solution develops a yellowish discoloration after about two weeks and must be discarded at that stage.

8. *Phosphorus Stock Standard.* Precisely 438.1 mg. of potassium dihydrogen phosphate are placed into a 100 ml. volumetric flask. Water is added to the mark. Twenty ml. of the stock standard are diluted to 1000 ml. with distilled water for use as a working standard. Each ml. of the working standard contains 0.02 mg. of phosphorus, and is equivalent to 0.645 mEq. of magnesium per liter of serum.

PROCEDURE

Into a 12-ml., fine-tipped, centrifuge tube are pipetted 2 ml. of serum, 2 ml. of distilled water, and 1 ml. of ammonium oxalate reagent. The contents of the tube are mixed and allowed to stand for one hour. The tube is centrifuged for ten minutes at 2500 r.p.m., and the supernatant is decanted into a second centrifuge tube with care not to disturb the precipitate of calcium oxalate. The precipitate is broken up by blowing in 2 ml. of 2 per cent ammonium hydroxide. The tube is re-centrifuged and the supernatant is again decanted into the second centrifuge tube. Suspension of the precipitate in 2 per cent ammonium hydroxide, centrifugation, and decantation are repeated once again. The precipitated calcium oxalate may be saved for determination of serum calcium by the Clark-Collip procedure. The combined supernatants contain the serum magnesium

To the combined supernatants are added 1 ml. of potassium dihydrogen phosphate reagent, and 1 ml. of concentrated ammonium hydroxide. The contents of the tube are mixed thoroughly and precipitation of magnesium ammonium phosphate is initiated by scratching the inside of the tube with a sharply pointed glass rod. The glass rod is rinsed with several drops of distilled water. The tube is stoppered and allowed to stand overnight in the refrigerator. The tube is then centrifuged at 2500 r.p.m. for thirty minutes. The supernatant is carefully decanted and discarded. Five ml. of chilled, alcoholic wash solution are added, and the tube is tapped in order to re-suspend the precipitate. The tube is re-centrifuged at 2500 r.p.m. for thirty minutes, and the supernatant is again discarded. Five ml. of distilled water are added, and the supernatant is again discarded. precipitate. A blank and two standards are prepared by pipetting 0, 2 and 4 ml. of phosphate working standard into graduated centrifuge tubes. The contents of these tubes are diluted to 5 ml. with distilled water.

To each tube are added 0.5 ml. of ammonium molybdate reagent and 0.4 ml. of aminonaphtholsulfonic acid reagent. The contents of the tube are diluted to 10 ml. and allowed to stand for five minutes. Measurements of optical density are made at 660 mμ.

CALCULATIONS

1. In a molecule of $MgNH_4PO_4$, 31 mg. of phosphorus are equivalent to 24 mg of magnesium.

2. The working phosphate standard contains 0.02 mg. phosphorus per ml.
   a. Thus, the 2 ml. standard represents 1.29 mEq. Mg per liter
   b. The 4 ml. standard represents 2.58 mEq. Mg per liter

$$1000 \text{ ml} \times \frac{0.04 \text{ mg. P}}{2 \text{ ml.}} \times \frac{24 \text{ mg. Mg}}{31 \text{ mg. P}}$$

$$\times \frac{1 \text{ mEq. Mg}}{12 \text{ mg. Mg}} = 1.29 \text{ mEq. Mg per liter}$$

3. A calibration curve should be prepared by plotting optical density versus Mg concentration in mEq per liter. The calculation may also be made using the nearest standard. Thus for the 4 ml. standard:

$$\text{Magnesium (mEq. per liter)} = \frac{\text{O.D. Unknown}}{\text{O.D. Standard}} \times 2.58$$

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C. Bohuon Method for the Measurement of Magnesium in Serum

JON V. STRAUMFJORD, JR., M.D., Ph.D., and BASIL DOUMAS, Ph.D.

INTRODUCTION

Numerous published methods for serum magnesium support the axiom that satisfaction in methodology is inversely proportional to the number of different methods. Some authors have felt that technical difficulties have been responsible for the confusion in the clinical interpretation of serum magnesium determinations, while others have indicated that clinical interpretation has been clouded by the predominantly intracellular distribution of magnesium. Agreement obtained in the analyses of normal serum with multiple methods would tend to support the latter view[1]. Many of the described methods are unsuitable for clinical laboratories because they are time-consuming or require instrumentation not readily available. There is a need for a simple, quick, yet suitably precise method for routine clinical laboratories. The method proposed by Bohuon[2] fulfills these criteria and does not require equipment beyond that usually found in clinical laboratories.

PRINCIPLE

This method utilizes a reagent discovered by Mann and Yoe[3]. Magnesium, in an aqueous alcohol medium at pH 9-10, changes the reagent color from blue to red. The change in absorption is sufficient for detection with spectrophotometers or colorimeters with a relatively wide band-pass.

REAGENTS

1. *Distilled water.* All water used for reagents and the procedure should be glass-distilled twice.

2. *Sodium tungstate 10 per cent (w/v).* Ten (10) gm. of $Na_2WO_4 \cdot 2H_2O$ (reagent grade—according to Folin) are added to approximately 75 ml. of water in a 100 ml. volumetric flask. The contents are mixed and diluted to 100 ml. The tungstate solution should be neutral or very slightly alkaline to phenolphthalein. The pH of the solution may be corrected by the addition of either base or acid[4]. However, if more than 0.4 ml. of 0.1 *M* NaOH is required to neutralize the tungstate solution, it should be discarded.

3. *Sulfuric acid, 0.667 N.* Concentrated H_2SO_4, reagent grade, 18.7 ml., is added with care to 700 ml. of water, mixed, cooled and diluted to 1000 ml. The acid solution is adjusted to 0.667 *M* following titration with standard base (1.0 *M* NaOH).

4. *Mann and Yoe Reagent*[*] Twenty-five (25) mg. of 1-azo-2-hydroxy-3- (2, 4-dimethylcarboxanilido)-napthalene-1′-(2-hydroxybenzene-5-sulfonate) are added to 200 ml. of absolute ethanol, heated gently to dissolve, cooled and diluted to 250 ml. with absolute ethanol. This reagent is stable for more than two months.

5. *Sodium borate, 0.08 M.* Sodium borate decahydrate reagent grade, 30.51 grams, is added to 500 ml. of hot water, cooled and diluted to 1000 ml. This reagent is stable for more than two months.

6. *Absolute ethyl alcohol.*

7. *Magnesium stock standard, 100 mEq./L.* Clean magnesium ribbon, 1.216 gm. is transferred to a one-liter volumetric flask. Approximately 100 ml. of distilled water and 16 ml. of concentrated hydrochloric acid are added. The magnesium is allowed to dissolve, then is diluted to volume with distilled water.

Working standards are prepared by diluting aliquots of the stock standard to 1.0 liter. Each milliliter of stock standard diluted to 1.0 liter is equal to 1.0 mEq. per liter. Therefore, standards equivalent to 1, 2, 3, and 4 mEq. per liter are prepared by diluting 1, 2, 3, and

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[*] Available from the LaMotte Chemical Products Company, Chestertown, Maryland, as Mann and Yoe Magnesium Reagent.

4 ml. of stock standard to 1.0 liter respectively.

PROCEDURE

Serum: Into a 15 ml. test tube are placed 0.5 ml. of serum and 3.5 ml. of water. The tube contents are mixed, and 0.5 ml. of 10 per cent sodium tungstate is added with thorough mixing, and is followed by 0.5 ml. of 0.667 N H_2SO_4. The tube contents are remixed, and the precipitated protein is separated by centrifugation. One (1.0) ml. of the supernatant fluid is removed and is placed into another 15-ml. test tube. One (1.0) ml. of water is added to the aliquot of the supernatant solution, mixed, and followed by 3 ml. of the Mann and Yoe reagent. The tube contents are mixed and 2.0 ml. of the 0.08 M sodium borate are added, and the contents are remixed. To the mixture, 3.0 ml. of absolute alcohol are added, remixed and set aside for twenty minutes for color development. The solution is transferred to a suitable cuvet, and the absorption is measured at 505 millimicrons. The concentration of the unknown is obtained from a predetermined standard curve using four working standards with concentrations of 1, 2, 3, and 4 mEq. per liter. The reagents and techniques are evaluated with each analysis by comparing two standards with the standard curve.

Blank: The blank is prepared by using 2.0 ml. of distilled water, followed by the same quantities of the Mann and Yoe, borate, and alcohol reagents as outlined above.

Standards: Standards are prepared by adding 1.0 ml. of the working standards, followed by 1.0 ml. of water and the color reagents as outlined in the procedure for serum.

DISCUSSION

This procedure provides good precision, is simple and well suited for routine laboratory use. The method, as originally described by Bohuon, can be used without prior protein precipitation. However, interference was reported for certain dysproteinemias and hyperbilirubinemias. Since this is a potential source of error, a deproteinization step should be used. The procedure is approximately eight times more sensitive than that employing titan yellow and appears to be suitably specific for magnesium[2]. Calcium does not significantly interfere with the reaction[2, 3]. Although the Klett-Summerson or Coleman, Jr., instruments are adequate for quantitation, the Beckman Model B or DU, or their equivalent is preferable.

The difference between the absorption peaks of the alcoholic reagent, with or without magnesium present is approximately 100 millimicrons. However, the method gives a high blank which is more troublesome with wide band-pass spectrophotometers. It is necessary to use a 10 or 12-mm. cuvette with the Coleman in order to adjust the instrument to 100 per cent transmission. The Klett-Summerson colorimeter has sufficient light energy to allow adjustment of the blank solution to zero absorption with a standard sized cuvette. Since the presence of magnesium decreases the absorption due to the reagent alone, the standardization curve does not intersect the origin unless a blank correction curve is constructed. But without the blank correction, the absorbance-concentration plot is linear from 0.5 to 3 mEq. per L., and thus, a correction curve is not necessary. The repeatability of this method is excellent. Coefficient of variation in the normal ranges is less than 3 per cent.

SOURCE OF ERROR

The principal sources of error with this technique are the usual ones, such as calculation mistakes, improper calibration of pipets and spectrophotometer contamination, etc. Since the absorption spectra are pH dependent, the amount of borate added to the color reagent is critical. Red cells contain approximately three times as much magnesium as serum; therefore, hemolysis may give spuriously high magnesium values. Ethylenediaminetetraacetate *(EDTA) in vitro* reverses the magnesium-reagent absorption peak to that of the blank. Thus, erroneously low values for serum magnesium may be expected from the presence of strong chelating agents of either a therapeutic or pathophysiologic source.

Range of Values for Normal Human Serum

	mEq/L	mg/100ml
Mean	1.8	2.2
Range	1.5-2.1	1.9-2.5

RESUMÉ OF CLINICAL INTERPRETATION

Specific symptomatology related to either hypo- or hypermagnesemia is difficult to ascertain because of other associated electrolyte abnormalities. Vallee, Wacker, and Ulmer[5] have described a magnesium deficiency syndrome with tetany. Hanna, Harrison, MacIntyre and Traser[6] have described a magnesium deficiency syndrome without tetany or abnormalities with other electrolytes. Although it would appear that syndromes related to magnesium deficiency exist, they are recognized relatively infrequently. The availability in clinical laboratories of quick, precise methods for serum magnesium measurements, and more extensive patient sampling may alter this observation.

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Measurements of Ultrafiltrable Calcium and Magnesium in Serum

F. WILLIAM SUNDERMAN, JR., M.D.

INTRODUCTION

Clinical interest in measurements of ultrafiltrable calcium and magnesium[4–8] has stimulated the development of precise methods for ultrafiltration[1, 11, 14, 17, 18, 20]. During the ultrafiltration of serum, it is necessary to maintain constant pH, and pCO_2, and to avoid evaporative losses. It has become recognized that the concentrations of calcium and magnesium in ultrafiltrates of serum may decrease with rise in pH and may increase with a rise in carbon dioxide tension[1, 7, 12, 14, 18]. A satisfactory and convenient technique for the preparation of serum ultra-filtrates is a modification of the method of Ames and Sakanoue[1], which employs an ingeniously designed micro-ultrafiltration apparatus.

Although the cation-binding properties of the serum proteins vary under certain pathologic conditions[13], estimations of the concentrations of serum ultrafiltrable calcium and magnesium may be computed from measurements of total serum calcium and magnesium by the McLean-Hastings[9] and Copeland-Sunderman[3] equations. Computations of ultrafiltrable calcium based upon measurements of serum albumin, globulins, and calcium have been reported by Leighton *et al.*[7]. Nomograms[7, 9, 19] based upon these equations, are included in this chapter.

Procedure for Ultrafiltration

PRINCIPLE

Blood is collected anerobically, and serum is separated without exposure to air. An ultrafiltrate of serum is prepared, with precautions to avoid alterations in pCO_2 and pH. In addition to measurements of calcium and magnesium in serum and ultrafiltrate, measurements are made of serum specific gravity or total proteins, so that the concentrations of calcium and magnesium may be expressed in terms of serum water.

APPARATUS

1. *Ultrafiltration apparatus,* of Ames and Sakanoue[1], as illustrated in Figure 1. This apparatus may be purchased from Mr. C. W. French, 15 Peterson Road, Natick, Massachusetts.

2. *Cellophane tubing,* Visking, 9/16″ diameter.

3. *Gas regulator valve,* with an attachment for saturating the gas with water vapor (National Cylinder Gas Co. Regulator #6486 M–#24703 S).

4. *Cylinder of gas* containing a mixture of 5 per cent carbon dioxide and 95 per cent oxygen, prepared by water pumping, (not by oil pumping).

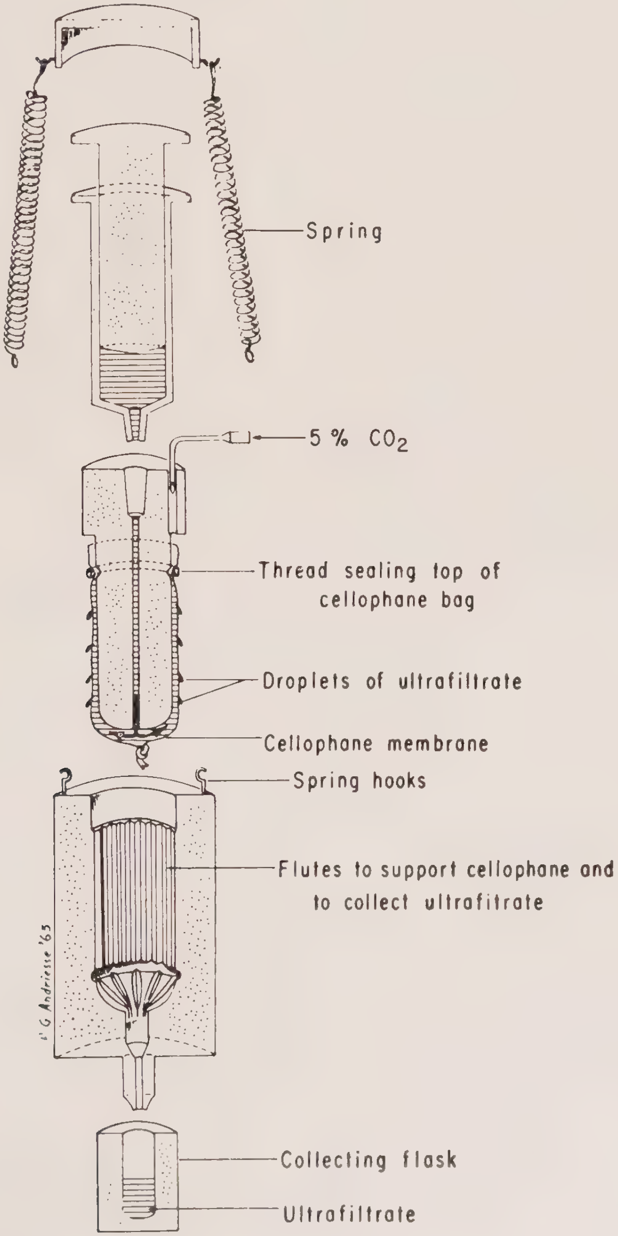

Spring
5 % CO_2
Thread sealing top of cellophane bag
Droplets of ultrafiltrate
Cellophane membrane
Spring hooks
Flutes to support cellophane and to collect ultrafitrate
Collecting flask
Ultrafiltrate
© G Andriesse '63

Fig. 1. Ames-Sakanoue Apparatus for ultrafiltration of serum (reproduced from Ames A., and Sakanoue, M.: J. Lab. Clin. Med. 64:168-176, 1964).

PROCEDURE

A segment of cellophane tubing is fitted snugly over the Lucite cylinder of the dialysis apparatus and is knotted at one end. The cellophane tubing is tied to a groove near the upper end of the cylinder with ten turns of #000 braided surgical silk. After the cellophane has been attached to the cylinder and the assembly has been placed into the fluted collecting chamber, the apparatus is flushed for five to ten minutes with a mixture of five per cent carbon dioxide and ninety-five per cent oxygen, saturated with water vapor.

Blood is collected anerobically and is allowed to clot under mineral oil in Pyrex tubes. With anerobic precautions, 4 ml. of serum are drawn into a syringe, and the syringe is attached to the ultrafiltration apparatus. To avoid damage of the cellophane tubing, the tubing is allowed to become moistened with serum for several minutes before attaching the springs or rubber bands which create a pressure gradient for ultrafiltration.

Before ultrafiltration is begun, the gas space within the apparatus is flushed briefly with the mixture of 5 per cent carbon dioxide and ninety-five per cent oxygen saturated with water vapor. Every fifteen minutes during ultrafiltration, the apparatus is flushed with the same gas mixture in order to maintain constant pH and pCO_2, and to aid in transferring the ultrafiltrate into the collecting flask.

With a pressure gradient of one atmosphere, the rate of ultrafiltration is 0.2 to 0.4 ml. per hour.

COMPUTATIONS

The concentrations of calcium and magnesium are measured in serum and ultrafiltrate. The specific gravity of serum at 20° C. is measured with a micropyknometer. Alternatively, the concentration of total serum protein is measured, and the specific gravity is computed from the equation of Moore and Van Slyke (10).

$$\text{Specific Gravity} = \frac{\text{Total protein (gm./100 ml.)} + 345.4}{343}$$

In the following table are given the factors by which, for any serum specific gravity, the concentration of solute per 100 ml. of serum may be converted to

Sp. Gr. 20°/20°	Multiplying Factors
1.015	1.035
1.016	1.038
1.017	1.040
1.018	1.043
1.019	1.045
1.020	1.048
1.021	1.051
1.022	1.053
1.023	1.056
1.024	1.059
1.025	1.061
1.026	1.064
1.027	1.067
1.028	1.069
1.029	1.072
1.030	1.075
1.031	1.078
1.032	1.081
1.033	1.083
1.034	1.086

the concentration of solute per 100 ml. of serum water[15, 16].

The concentrations of solute in the protein-free ultrafiltrate are converted to the concentrations of solute per 100 ml. of ultrafiltrate water by use of a multiplication factor of 1.007. Correction is thereby made for the volume which total solids occupy in the protein-free fraction[2, 10].

The percentages of ultrafiltrable calcium and magnesium are computed by the following equations:

$$\text{Ultrafiltrable Ca (per cent)} = \frac{\text{Ca in ultrafiltrate (mg./100 ml. } H_2O)}{\text{Ca in serum (mg./100 ml. } H_2O)} \times 100$$

$$\text{Ultrafiltrable Mg (per cent)} = \frac{\text{Mg in ultrafiltrate (mg./100 ml. } H_2O)}{\text{Mg in serum (mg./100 ml. } H_2O)} \times 100$$

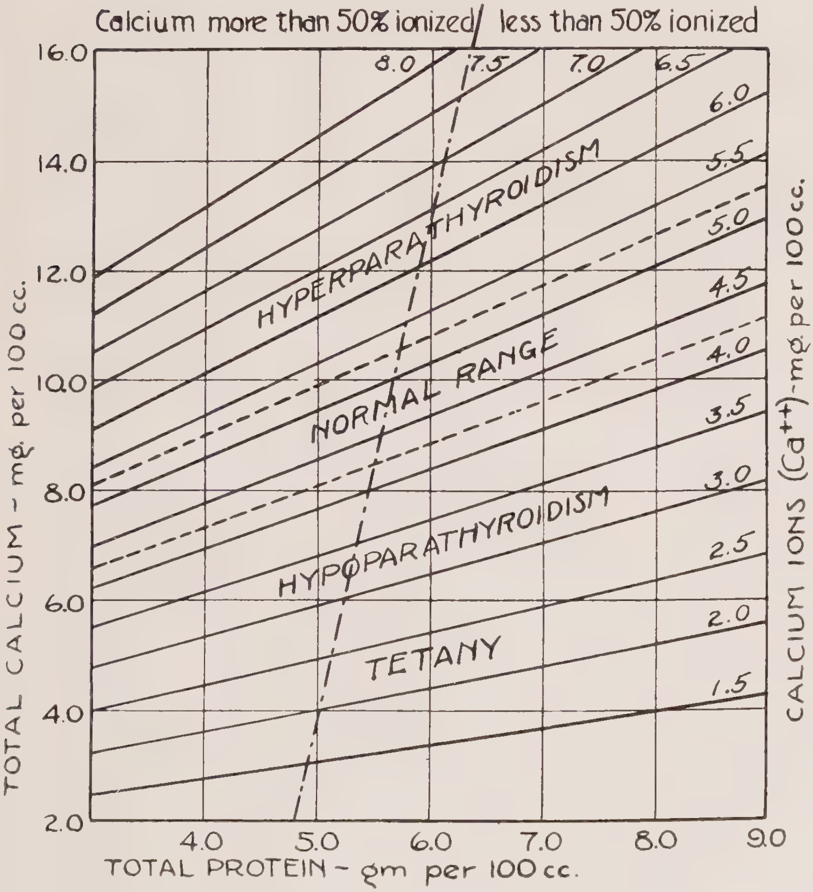


Fig. 2. Chart for calculation of Ca++ concentration from total protein and total calcium of serum or plasma (reproduced from McLean, F. C., and Hastings, H. B.: Amer. J. Med. Sci. 198:601-613, 1935).

NORMAL VALUES

Normal values for the percentages of ultrafiltrable calcium and magnesium in serum have been reviewed by Copeland and Sunderman[3], Munday and Maby[11], Prasad and Flink[12], and Toribara[18].

NOMOGRAMS

The McLean-Hasting nomogram[9] for calculations of ionized calcium in serum from measurements of total serum protein and total serum calcium is reproduced in Figure 2. The nomogram of Leighton and co-workers[7] for computation of ultrafiltrable calcium from measurements of serum albumin, globulin and total calcium is reproduced in Figure 3. The Willis-Sunderman nomograms[19] for calculation of concentrations of ionized magnesium in ultrafiltrates and serum from measurements of total serum protein and total serum magnesium are reproduced in Figures 4 and 5. These nomograms provide estimations of the partitions of calcium and magnesium in serum which are sufficiently accurate for most clinical purposes.

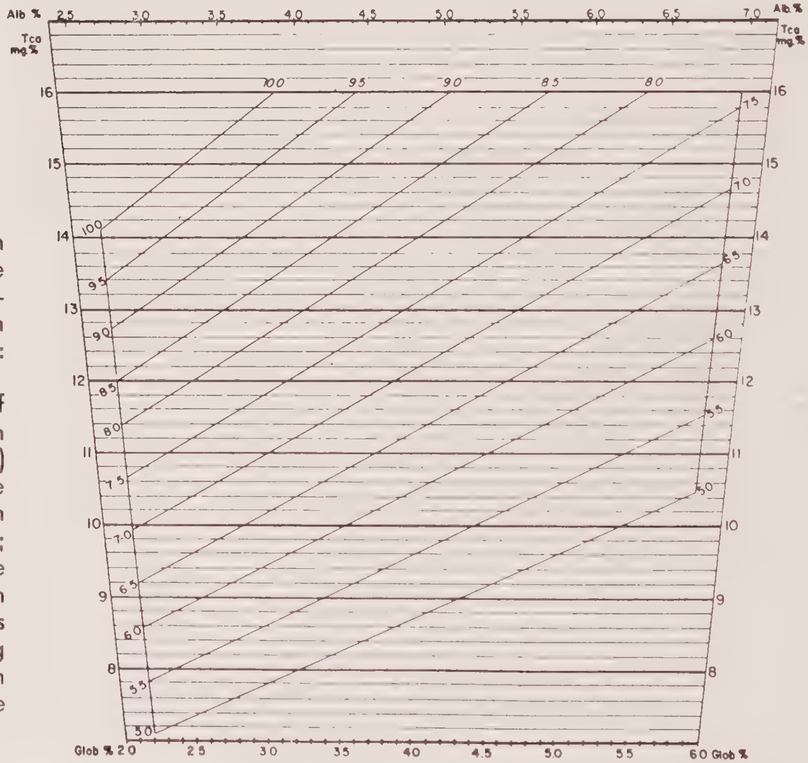
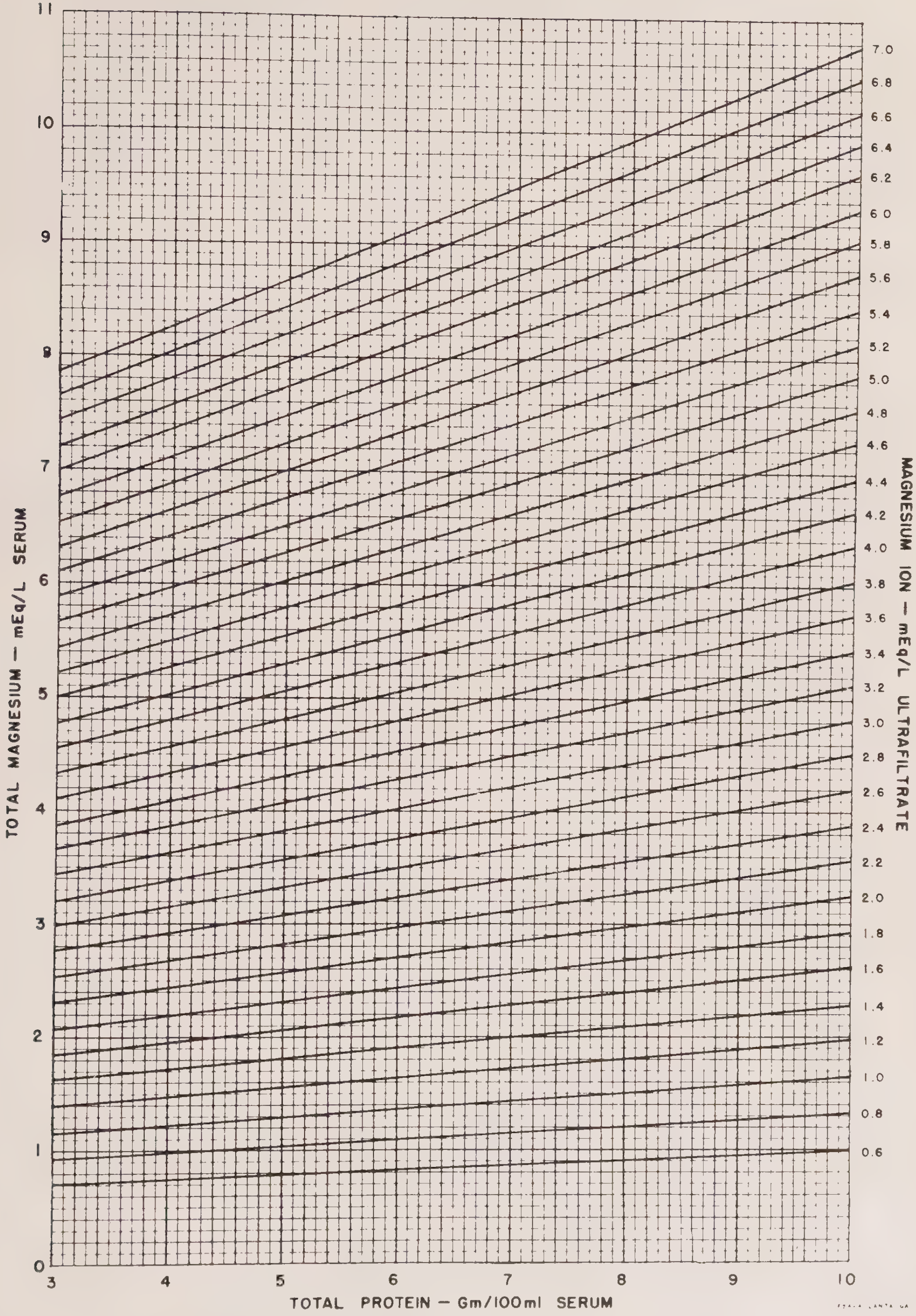


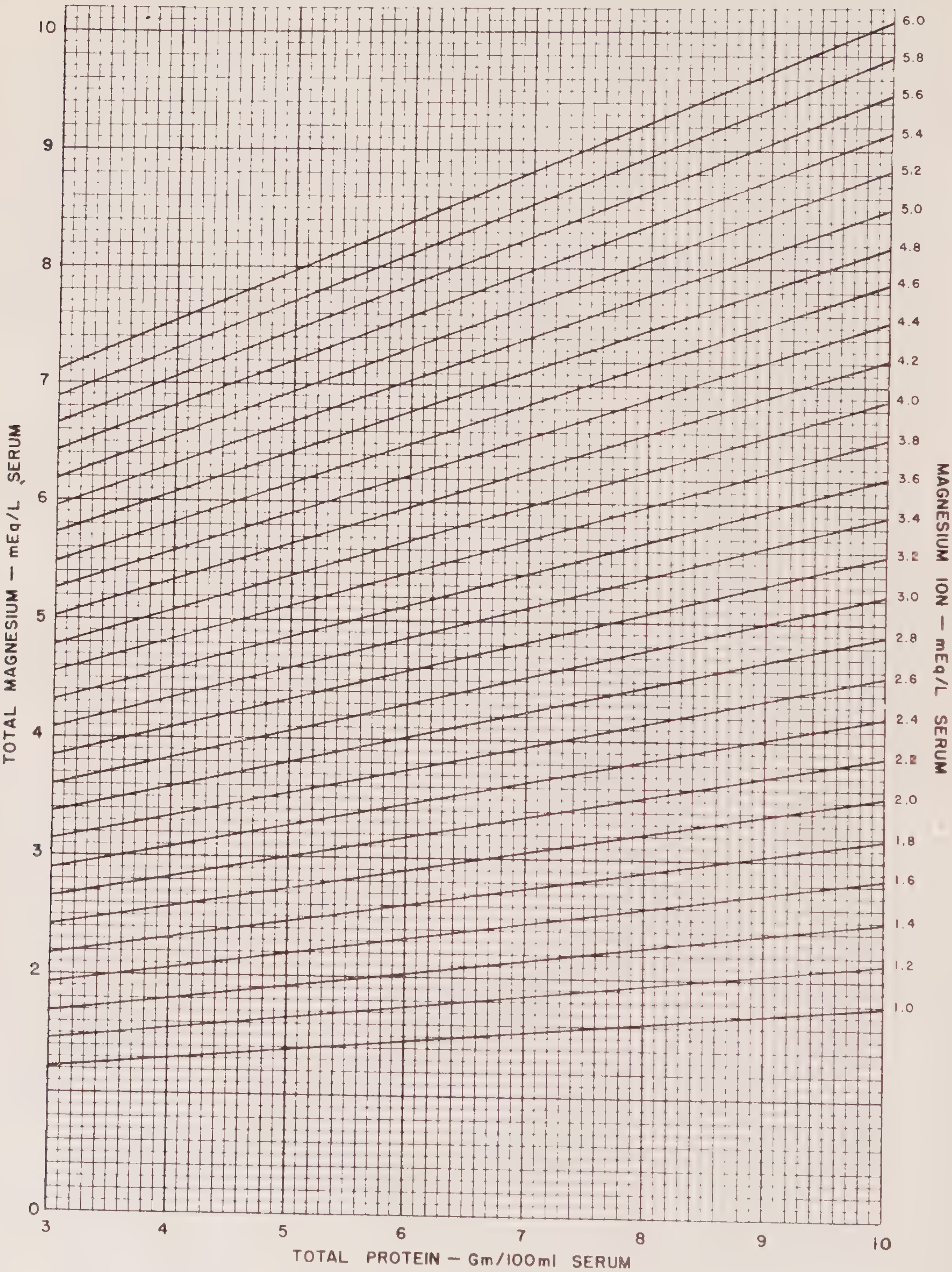
Fig. 3. Nomogram for estimating diffusible serum calcium (reproduced from Leighton et al., Clin Chem. 10: 235-240, 1964).

The concentration of serum diffusible calcium is computed by: (1) marking a straight line between the albumin and globulin values; (2) noting where the value for total serum calcium crosses this line, and (3) estimating the diffusible calcium by reference to the diagonal lines.



CALCULATION OF CONCENTRATION OF MAGNESIUM ION IN ULTRAFILTRATE FROM MEASUREMENTS OF TOTAL PROTEIN AND TOTAL MAGNESIUM IN SERUM

Fig. 4. Modified from Willis, M. J., and Sunderman, F. W.: J. Biol. Chem. 197:343-345, 1952.



CALCULATION OF CONCENTRATION OF MAGNESIUM ION IN SERUM FROM MEASUREMENTS OF TOTAL PROTEIN AND TOTAL MAGNESIUM IN SERUM

Fig. 5. Modified from Willis, M. J., and Sunderman, F. W.: J. Biol. Chem. 197:343-345, 1952.

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Measurement of Chloride in Biological Fluids

J. DE LA HUERGA, M.D., Ph.D., G. W. SMETTERS, M. D., and
JOSEPH C. SHERRICK, M.D.

A. General Considerations

The accurate measurement of chlorides in biological fluids is particularly important to the clinician in dealing with disturbances of electrolyte balance. The normal range of chloride in the serum is narrow, and specific, precise and reproducible methods of determination are essential. The measurement of chloride in urine, tissues, sweat, and stools may also be of clinical interest. For practical purposes, the methods of chloride measurement may be grouped into several distinct classes, and each method has several modifications.

THE VOLHARD TITRATION

Volhard devised the first practical technic for chloride measurement in serum and urine in 1874, following principles worked out by Mohr in 1856 and Gay-Lussac in 1833. In this method, chlorides are precipitated as silver chloride by adding an excess, but known amount, of silver nitrate[23, 40]. The amount of uncombined silver remaining is determined by titration with standard thiocyanate solution using ferric ion as indicator. In biological material, interfering proteins must first be eliminated, and this may be done either by the "open Carius" technic[15, 33, 35, 60, 68], or by applying the Volhard technic to protein free filtrate[42, 67]. The results with this technic compare well with those obtained by gravimetric methods after destroying protein with the Parr apparatus[23, 54]. Methods involving the Volhard technic are widely used, the chief disadvantage being fading of the indicator at the end point[47], due to the fact that silver chloride is more soluble than silver thiocyanate, and consequently, a slight excess of thiocyanate ion may combine with silver ion released from the precipitated silver chloride. This requires more thiocyanate reagent and thus produces false low chloride results. If the precipitated silver chloride is filtered, a further error is introduced due to the absorption of silver ion by the precipitate[32, 64]. In addition, the accurate preparation of several standard solutions is necessary.

IODOMETRIC METHODS

McLean and Van Slyke, in 1915, precipitated chloride in serum as silver chloride by the addition of silver iodate, leaving iodate ions in solution. The iodate may be measured gasometrically[50], titrametrically, by adding acidified potassium iodide and measuring the iodine evolved in the thiosulfate-starch reaction[29, 30, 50], or photometrically, by measuring the yellow color of the iodine evolved or the blue color formed with starch[30, 44, 49–53].

ADSORPTION INDICATOR METHODS

The original work in this field was done by Fajans and Hassel in 1923, using eosin and fluorescein as indicators. In methods of this type, such as the one described by Saifer and Kornblum in 1935, the chloride is titrated directly with silver nitrate in the presence of the adsorption indicator, dichlorofluorescein, which is adsorbed by the precipitate at the stoichiometric point, producing a colored compound[4, 19, 31, 45, 46].

MERCURIMETRIC METHODS

In 1853, Liebig described a method for determining chloride by mercuric nitrate titration. Dubsky and Trtílek[14] first used diphenylcarbazone as the indicator for this method in 1933, and Lang adapted the method to body fluids in 1937. The modification described by Schales and Schales in 1941 is presently widely used. The procedure is based on the titration of the chloride containing solution with a standardized acidic solution of mercuric nitrate. This results in the formation of soluble but undissociated mercuric chloride. When the chloride is all combined, the mercuric ions react with diphenylcarbazone to form a colored coordination complex at the end point. The end point may be difficult to determine in icteric serum specimens, and Fingerhut and Marsh have described a technic for destroying the bile pigments, using hydrogen peroxide and heat.

Gerlach and Frazier have studied the coordination complex occurring at the end point, and they find that it has an absorption maximum at 520mμ and a molar absorptivity of 19,000. The reaction follows Beer's law. These authors have adapted the procedure described by Clarke into a colorimetric mercurimetric procedure for the determination of chloride in serum.

MICRODIFFUSION METHOD

Conway (1947) described a method involving the oxidation of chloride ion to chlorine by a sulfuric acid-potassium permanganate mixture in the outer chamber of a Conway diffusion chamber. The liberated chlorine is absorbed by potassium iodide, and iodine is liberated; this may be estimated colorimetrically or titrated against sodium thiosulfate.

ELECTROMETRIC METHODS

Cotlove and associates have described an instrument for electrometric chloride titration based on established principles of coulometry[36]. A coulometric circuit generates silver ions which combine with chloride. An amperometric circuit indicates the first appearance of free silver ions at the end point and triggers a relay circuit to interrupt the titration and stop a timer. The elapsed time is proportional to the number of silver ions generated, which is proportional to the amount of chloride in the solution.

A null point-potientiometric method has been described by Malmstadt and Weinfordner (1959), a conductometric titration by Teloh (1954), and a polarographic method by Baginski and associates (1958).

COLORIMETRIC METHODS

In this group are included some methods already mentioned, such as photometric modifications of the iodometric, mercurimetric, and microdiffusion methods. Some colorimetric methods are known as displacement or exchange methods, since they involve displacement of a highly colored ion from a relatively insoluble compound by chloride ion, the last forming a less soluble or slightly dissociated salt. For example, silver chromate reacts with chloride ion to produce insoluble silver chloride and chromate ions. The yellow chromate solution may be measured directly[25] or the purple-red color formed by the reaction between chromate ions and diphenylcarbazide may be measured[37, 66]. The Fantus test for urine chlorides is based on a reaction of this type combined with a silver nitrate titration. Another indirect method involves the use of silver dithizonate as an exchange reactor for chloride ion. Solubilities favor the formation of silver chloride with liberation of colored dithizone[2, 6].

A third type of exchange reaction based on differential solubilities was described by Iwasaki and associates, in 1952, using mercuric thiocyanate. Chloride ions displace thiocyanate ions which are combined with ferric ions to produce brown ferric thiocyanate[7, 28, 70]. The ferric perchlorate method described by West and Coll (1956) is analogous.

A spectrophotometric method utilizing mercuric chloranilate, which reacts at low pH with chloride to form mercuric chloride and purple chloranilate ion, was described by Barney and Bertolacini in 1957. Adaptation of the method for chloride in serum was studied by Hausman and Baginski and associates in 1958, and by Baar in 1962. Itano *et al.* described a method for chloride in urine in 1959. Mercuric chloride is colorless, and the intensity of color of the purple chloranilate ion liberated is proportional to the concentration of chloride.

CHOICE OF METHOD

The basic reference method for measurement of chloride in the routine laboratory is the Wilson and Ball modification of the Volhard titration. This is a precise reproducible method which has been widely used and which has withstood the test of time. The principle disadvantages, as mentioned, are that it is time-consuming, requires titration, and that fading occurs at the end-point.

Perhaps the most commonly used chloride method is the Schales mercurimetric procedure. The only disadvantage is that careful microtitration is required, and titration is becoming less and less a routine skill of the average technologist.

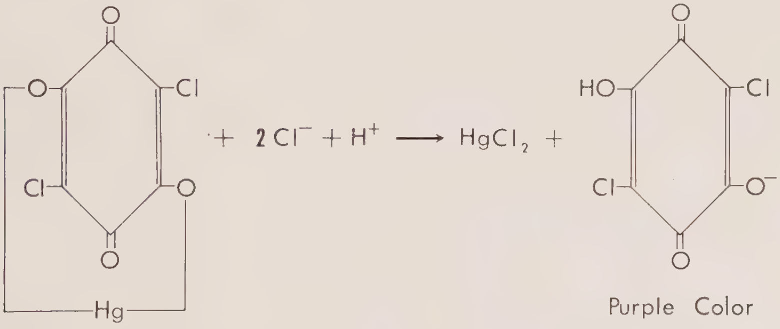
The electrometric methods are also widely used, but these require costly specialized apparatus and seem to produce results which are, in general, higher than those observed with the reference method.

A colorimetric method utilizing mercuric chloranilate has been recently studied in our laboratory, and has been found to be simple, rapid and reproducible. It is remarkably sensitive, which makes it suitable for ultramicro determinations and useful for measuring chloride in dilute solutions, as for example, sweat.

B. Mercuric Chloranilate Method for the Estimation of Chloride in Biological Fluids

PRINCIPLE

In acidic solution, chloride ion reacts with almost insoluble mercuric chloranilate to form colorless mercuric chloride and chloranilate ion, which is highly colored. The intensity of this color is proportional to the concentration of chloride and can be measured spectrophotometrically at 530 millimicrons. The reaction is as follows:



Mercuric Chloranilate

Chloranilate Ion

SERUM METHODS

Reagents

1. *Stock Deproteinizing Reagent.* Twenty grams of sodium tungstate are transferred to a one-liter volumetric flask and dissolved in about 600 ml. of distilled water. Three ml. of concentrated (syrupy) phosphoric acid are added and mixed. One hundred ml. of 5 N. nitric acid are added while mixing, and distilled water is added to the one liter mark. This reagent is stable at room temperature without precipitation of tungstic acid.

2. *Working Deproteinizing Reagent.* One volume of the stock deproteinizing reagent is diluted to ten volumes with distilled water.

3. *Stock Mercuric Chloranilate Reagent.* Two grams of mercuric chloranilate

are transferred to a 50-ml. centrifuge tube, and 40 ml. of approximately 0.05 N. nitric acid are added. The tube is stoppered, shaken and centrifuged, and the supernatant is discarded. This washing process is repeated until the supernatant is colorless, thus removing free chloranilate ion which may be present in some batches of mercuric chloranilate. The crystals remaining are resuspended in 200 ml. of 0.05 N. nitric acid, making a suspension of 10 mg. per ml. This reagent may be kept at room temperature. The supernatant liquid may become colored, but this does not affect the final result, since the supernatant is discarded in the preparation of the working reagent.

4. *Standard Sodium Chloride Solution, 100 mEq. per liter.* To a 1-liter volumetric flask are transferred 5.845 gm. of chemically pure dried sodium chloride. This is dissolved in about 500 ml. of distilled water, and the flask is filled to the mark with distilled water.

5. *Deproteinizing-chloranilate Reagent.* This reagent is made up on the day of use in a volume according to the number of procedures to be done. For each tube, 1.5 ml. of stock mercuric chloranilate (reagent 3) and 15 ml. of working deproteinizing reagent (reagent 2) are necessary. To make this reagent (reagent 5), the stock bottle of mercuric chloranilate is shaken. The proper volume is promptly withdrawn, centrifuged and the supernatant discarded. The crystals of mercuric chloranilate remaining are resuspended in the calculated volume of reagent 2, and the mixture is transferred to an Erlenmeyer flask and resuspended as necessary during the procedure.

PROCEDURE

I. *Routine Method (100 microliters of serum).*

1. Three 16 x 150 mm. test tubes are labeled "B," "S," "X," and to each is transferred exactly 15 ml. of reagent 5, properly mixed before each transfer.

2. Exactly 100 microliters of distilled water are added to tube "B," 100 microliters of 100 mEq. per liter standard to tube "S," and 100 microliters of serum to tube "X." The tubes are stoppered and shaken vigorously.

3. The test tubes are centrifuged for five minutes at high speed and the supernatant in each is transferred to a properly labeled 19-mm. Coleman cuvet.

4. With the spectrophotometer[*] adjusted to a wave length of 530 millimicrons, the galvanometer is adjusted to 100 per cent transmittance with the blank (tube "B"). Readings from the standard ("S") and unknown ("X") are obtained.

Calculations: After converting the readings to absorbancies, the chloride concentration is calculated by the following formula:

$$\text{Concentration of unknown (mEq. per liter)} = \frac{100 \text{ x absorbancy of unknown}}{\text{absorbancy of standard}}$$

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[*] The procedure was designed for the Coleman Junior Spectrophotometer in such a way that readings for 100mEq. per liter of chloride are close to 37 per cent transmittance, in the most accurate and sensitive range of the instrument. The method may be adapted to any spectrophotometer with appropriate adjustments for dilutions, concentration of reagents and size of cuvets.

Note: The procedure may be performed utilizing 50 μl. of serum and 7.5 ml. of deproteinizing-chloranilate reagent (reagent 5), with satisfactory results.

II. *Ultra Micro-Method (20 Microliters of serum).*

1. To each of three appropriately labeled 10 x 75 mm. test tubes are transferred 2.0 ml. of deproteinizing-chloranilate reagent. Twenty microliters of distilled water, serum, or standard are added to the appropriate tube.

2. After mixing and centrifuging, readings are obtained from the supernatant as in the routine method, except that 12 mm.-Coleman cuvets are used.

3. Calculations are performed in the same manner.

III. *Method for the Measurement of Chloride in Sweat.*

The recommended method of collecting sweat is by iontophoresis[55]. Since the chloride content of sweat is lower than serum, certain minor changes in technic are necessary.

Reagents

The reagents are those described for the routine procedure.

Procedure

1. The sweat is collected and weighed in the usual manner.

2. Eight ml. of deproteinizing-chloranilate mixture (reagent 5) are added to the container of sweat. Eight ml. of deproteinizing-chloranilate reagent (reagent 5) are added to each of two similar containers, each of which has in it a gauze pad or filter paper like that used to collect the sweat, but containing no sweat. One of these containers is labeled "B" (blank), the other "S" (standard).

3. Fifty microliters of distilled water are added to the container labeled "B," and 50 microliters of standard (reagent 4) are added to container labeled "S."

4. After shaking for thirty seconds, the contents of each container are transferred to properly labeled centrifuge tubes, and centrifuged at high speed for five minutes.

5. The supernatant fluids are transferred to properly labeled 19-mm. Coleman cuvets, and per cent transmittance readings at 530 millimicrons are obtained, using the contents of "B" to adjust the spectrophotometer to 100 per cent transmittance.

Calculations: After converting the per cent transmission readings to absorbancies, the chloride concentration is calculated by the following formula:

$$\underset{\text{(mEq./L)}}{\text{Concentration unknown}} = \frac{5000 \times \text{absorbancy of unknown}}{\text{Wgt. sweat in mg.} \times \text{absorbancy of standard}}$$

IV. *Urine Method.*

Although the amount of chloride in the urine may vary widely, it generally contains somewhat less chloride than does serum.

Reagents

The reagents described in the routine procedure are used.

Procedure

1. Three 16 x 150 mm. test tubes are labeled "X," "S," "B," and to each are added 15 ml. of deproteinizing-chloranilate reagent (reagent 5). Two hundred microliters of urine are added to tube "X," and 200 microliters of distilled water are added to tube "B." One hundred microliters of standard and 100

microliters of water are added to tube "S."

2. After shaking, the tubes are centrifuged and the supernatant transferred to appropriately labeled 19-mm. Coleman cuvets.

3. Readings are obtained in the same manner as for the routine determination.

CALCULATION

$$\underset{\text{(mEq. per liter)}}{\text{Concentration of chloride}} = \frac{50 \text{ x absorbancy of unknown}}{\text{absorbancy of standard.}}$$

NOTES

The results should be reported as mEq. per 24 hours when the 24-hour urine volume is known. If the reading of the unknown is less than 10 per cent transmittence, the contents of cuvet "X" may be diluted with the blank and read directly with appropriate changes in the calculation.

C. Mercurimetric Method for the Estimation of Chloride in Serum

PRINCIPLE

Chloride ions in serum are titrated with a standardized acidic solution of mercuric nitrate, using the appearance of a violet-blue color produced by an excess of mercuric ions with diphenylcarbazone as the end point.

$$2\,Cl^{-} + Hg^{++} \rightarrow Hg\,Cl_2$$

$$Hg^{++} + \text{diphenylcarbazone} \rightarrow \text{Hg-diphenylcarbazone complex (violet-blue color).}$$

REAGENTS

1. *Standard Sodium Chloride Solution, 100 mEq. per Liter.* This is prepared as described for the mercuric chloranilate method.

2. *Mercuric Nitrate Reagent.* Two hundred ml. of distilled water are transferred to a 1-liter volumetric flask and 2.5 ml. of concentrated nitric acid are added. Mercuric nitrate monohydrate ($Hg(NO_3)_2 \cdot H_2O$), 1.87 gm., is added and dissolved, and the flask is filled to the mark with distilled water. After mixing, the reagent is titrated with the chloride standard, using the same technic as described for the determination of chloride in serum. The reagent should be diluted with water so that 1.67 ml. of the mercuric nitrate reagent will combine with chloride present in 200 microliters of 100 mEq. per liter standard chloride solution. For example: If the titrating figure was 1.63 ml., then $\frac{(1.67 - 1.63) \times 1000}{1.67} = 23.0.$ In this case, 23.0 ml. of water are transferred to a 1-liter volumetric flask, and

the flask is filled to the mark with the mercuric nitrate reagent. After mixing, the reagent should be titrated against the standard sodium chloride solution, and the chloride factor of the reagent calculated as follows: $\frac{100}{\text{ml. used in titration}}$ The reagent is stable, but it is advisable to check the titration factor once a month.

3. *Chloride Indicator.* Fifty mg. of S-diphenylcarbazone are dissolved in 50 ml. of ethyl alcohol. This reagent is placed in a brown dropper bottle. The reagent should be yellow. It should be made up fresh when it turns cherry pink, or about once a month.

4. *Dilute Nitric Acid (0.2 per cent V/V).* One ml. of concentrated nitric acid is diluted with distilled water to make 500 ml.

PROCEDURE

1. To a small beaker, 1.5 ml. of 0.2 per cent nitric acid is added.
2. Exactly 200 microliters of serum are added.
3. A small magnetic stirrer is placed in the beaker, and the beaker is placed on a magnetic plate. The rotation of the stirrer is adjusted to a speed which will mix the contents without spilling them.
4. Three or four drops of chloride indicator are added.
5. Using a 2 ml. microburet filled to the zero level with mercuric nitrate reagent, titration is begun and is continued until the solution in the beaker turns a pink color which persists for at least one minute.
6. The number of ml. of standard mercuric nitrate solution used is recorded.

CALCULATION

Chloride concentration (mEq./L.) = ml. used in titration × chloride factor of mercuric nitrate reagent.

Note: If the serum sample is jaundiced the end point will be difficult to detect, and it is necessary to perform the determination on a protein-free filtrate of the sample.

D. Argentimetric Method for the Measurement of Chloride in Biological Materials

PRINCIPLE

The chlorides in serum are precipitated by an excess but known amount of silver nitrate. Nitric acid and heat are used to digest the proteins. The excess silver nitrate is titrated with ammonium thiocyanate in the presence of ferric ion, and a white precipitate of silver thiocyanate is formed. The end point is indicated by the appearance of the red color produced by an excess of thiocyanate ions with ferric ion.

$$AgNO_3 + Cl \rightarrow AgCl\downarrow + NO_3^-$$
$$AgNO_3 + CNS^- \rightarrow AgCNS\downarrow + NO_3^-$$
$$3\ CNS^- + Fe^{+++} \rightarrow \underset{\text{red color}}{Fe\,(CNS)_3}$$

REAGENTS

1. *Silver Nitrate Solution.* Twenty-six grams of silver nitrate are weighed and dissolved in approximately 500 ml. of water in a 1-liter volumetric flask. Distilled water is added to the mark.

2. *Ferric Ammonium Sulfate Indicator.* Five grams of $Fe(NH)_4\ (SO_4)_2 \bullet 12\ H_2O$ are dissolved in 100 ml. of water.

3. *Nitric Acid, Concentrated c.p.*

4. *Ammonium Thiocyanate Solution.* One and one-half grams, of NH_4CNS is weighed, dissolved, and diluted with distilled water to 1000 ml. in a 1-liter volumetric flask.

5. *Standard Sodium Chloride Solution 100 mEq. per liter.* This solution is prepared as described for the mercuric chloranilate method.

PROCEDURE

1. Three 25 x 150 mm. digestion tubes are labeled "X," "S," and "B." Exactly 1 ml. of serum is added to "X," 1 ml. of distilled water to "B," and exactly 1 ml. of chloride standard to "S."

2. Exactly 1 ml. of silver nitrate solution (reagent 1) is added to each of the tubes, drop by drop, with constant mixing.

3. Three ml. of concentrated nitric acid are added to each tube and the contents mixed.

4. The mixture is digested by placing the tubes in a boiling water bath for one hour, or until the proteins have been completely dissolved and a clear yellow supernatant liquid remains.

5. Six ml. of ferric ammonium sulfate indicator are added to each tube and the solution is cooled to room temperature.

6. The solution in each tube is titrated with ammonium thiocyanate solution using a 10 ml. buret graduated in 0.02 ml. The titration is continued until one drop of thiocyanate solution causes a color change which persists for about one minute.

CALCULATION

$$\underset{\text{(mEq per liter)}}{\text{Concentration of chloride}} = \frac{100(\text{TB-TX})}{\text{TB-TS}}$$

TB = ml. thiocyanate used for blank.
TX = ml. thiocyanate used for unknown.
TS = ml. thiocyante used for standard.

NOTES

a. Accurately weighed silver foil may be used to standardize the silver nitrate reagent, from which the ammonium thiocyanate reagent may be standardized. This method of standardization is precise but cumbersome, and it is considered that the use of sodium chloride solution as a primary standard is just as satisfactory.

b. *Urine:* The argentimetric method may be used for measurement of chloride in urine. In most cases, urine contains no protein, and the digestion by heat may be omitted if the tubes are placed in the dark for five minutes. If the concentration of chloride in the urine is unusually high, it may be necessary to use 0.5 ml. of urine instead of 1.0 ml., in which case the results are multiplied by two. If results are low, 2 ml. of urine may be used and the results divided by two.

c. *Tissue:* The argentimetric procedure is satisfactory for measurements of chloride in tissues provided the material is first subjected to alkaline hydrolysis according to the suggestion of Sunderman and Williams[56–58]. Other methods of chloride measurement in tissues are described by Dean and Fishman (1941) and Weinstein and Jennings (1959).

E. Discussion of Chloride Methodology

MERCURIC CHLORANILATE METHOD

Because of the relatively small variation in the serum chloride content of normal individuals, special precautions must be taken to obtain results reproducible within ± 2 per cent. The micropipets should be carefully calibrated and the cuvets perfectly matched. The glassware, distilled water, and reagents must be free of chlorides. At least one standard should be assayed with each batch of determinations, and it is desirable to include one known serum sample for quality control.

Although Baar[1] reported no difficulty in performing the determination by adding solid mercuric chloranilate directly to a diluted serum sample, definite turbidity was encountered in many serum samples when this technic was followed in our laboratory.

Consistently better results were obtained when the determination was performed on a protein-free filtrate of serum. It was decided to use a combined reagent incorporating both the deproteinizing reagent and chloranilate reagent after it was demonstrated that entrappment of chlorides in the precipitated protein did not occur with such a mixture. In experiments designed to clarify this point, no difference in chloride results could be detected, whether the reagents were combined or used separately. It did not matter whether the mercuric chloranilate was added to the protein-free filtrate or to the solution after proteins were precipitated, but before they were removed by filtration. Thus, the use of the combined reagent was justified.

In the routine procedure the deproteininzing reagent will extract all the protein from samples containing as much as 33 grams of protein per 100 ml. (22 grams per 100 ml. in the ultramicro procedure). Theoretically, under the conditions of the procedure, sufficient mercuric chloranilate is present to combine with all the chloride in solutions containing at least 750 mEq. per liter in the routine procedure (500 mEq. per liter in the ultramicro procedure). The combined reagent contains one mg. of mercuric chloranilate per ml.; varying this amount from 0.5 to 4.0 mg. per ml. produced no change in color intensity.

The pH of the medium is 1.3. Changes in the concentration of nitric acid from 0.02 N. to 0.085 N. did not produce any change in color intensity. With higher concentrations of nitric acid the amount of color produced was less and the blanks were more highly colored.

Investigations carried out with this procedure have shown that linearity is excellent up to 150 mEq. per liter. If the color is too intense to read directly in the spectrophotometer, accurate results may be obtained by diluting the final reagent with reagent blank.

Bromide ions react with mercuric chloranilate in the same manner as chloride ions; therefore, a high level of serum bromides may produce inaccurate results. This same lack of chloride specificity applies equally to the other methods. According to Baar, sulfates, phosphates, iron, and copper do not interfere with the reaction at physiological levels. Among commonly used anticoagulants only *EDTA* caused interference. Our investigations indicate that fluoride neither enters into nor interferes with the reaction.

OTHER METHODS

The mercurimetric and argentimetric methods have been extensively studied,

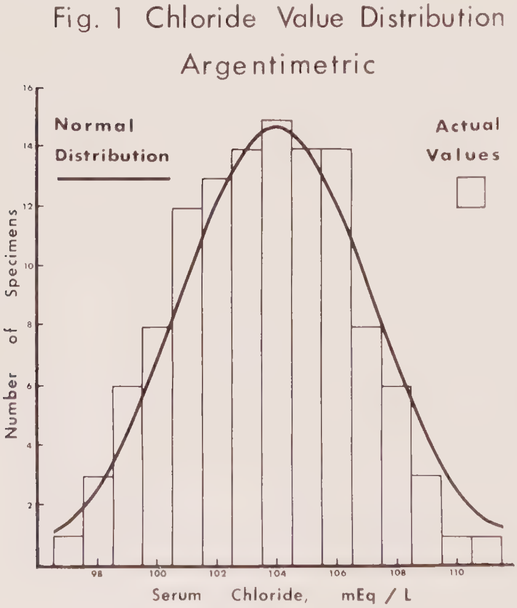

Fig. 1. Frequencies of occurrence of serum chloride values determined by argentimetric method (hollow bars) compared to normal frequency distribution curve (solid line).

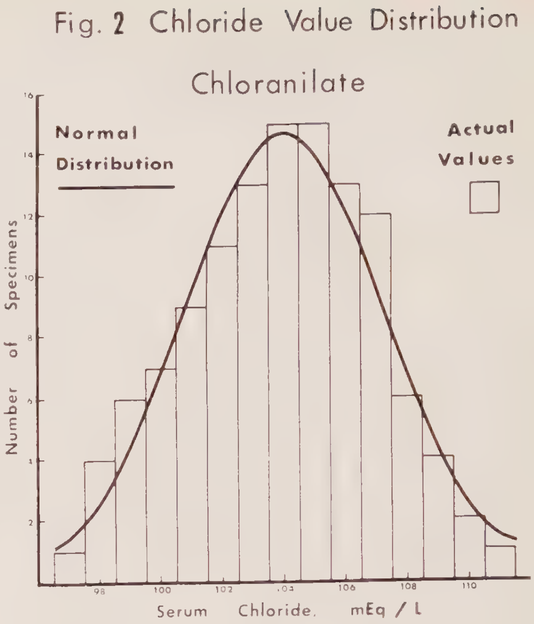

Fig. 2. Frequencies of occurrence of serum chloride values determined by chloranilate method (hollow bars) compared to normal frequency distribution curve (solid line).

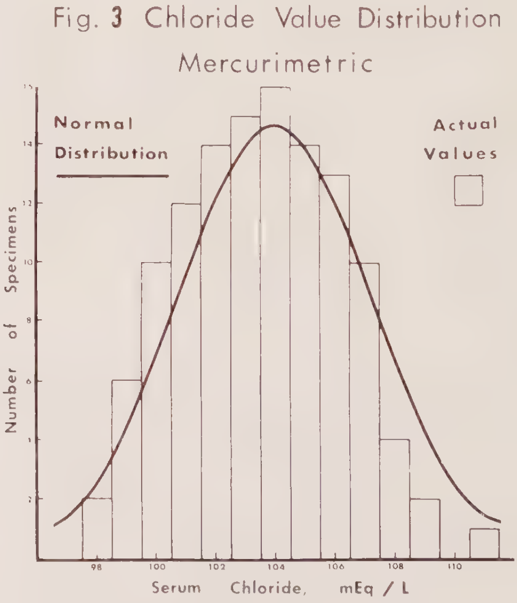

Fig. 3. Frequencies of occurrence of serum chloride values determined by mercurimetric method (hollow bars) compared to normal frequency distribution curve (solid line).

and additional information may be obtained from the references[15, 17, 23, 32–33, 35, 38, 40, 42, 47, 55–58, 60–62, 67–68].

NORMAL VALUES

Serum samples from 119 adults without known abnormality were analyzed by the three methods. Figures 1-3 show the frequency distribution of values obtained by the three methods compared to the normal distribution curve expected theoretically. They demonstrate that serum chloride levels in this population followed a Gaussian normal distribution curve when determined by any of the three technics studied. Because the frequencies are normally distributed, it is possible to specify the range of values as means with standard deviations[69], as shown in Table I.

TABLE I

METHOD	MEAN mEq/L	STANDARD DEVIATION	RANGE* mEq/L
Argentimetric	103.8	3.2	97.4-110.2
Chloranilate	104.0	3.1	97.8-110.2
Mercurimetric	104.1	3.3	97.5-110.7

*Range = Mean ± 2 standard deviations.
Chi-square values were: argentimetric 4.18, chloranilate 3.52, mercurimetric 9.38.

Our studies on the chloride content of sweat and urine have been limited, but they are in agreement with the results of others.

According to di Sant'Agnese, the normal range for sweat chlorides is from 0 to 50 mEq./L., with a mean of 19 mEq./L. In fibrocystic disease of the pancreas, the mean is 102 mEq./L., with a range of 50 to 180 mEq/L. Fifty mEq./L. is generally taken as the upper limit of normal[60]. Individual variations from day to day may be wide.

The normal range for urine chloride on a normal diet is usually considered to be 170 to 250 mEq./24 hours[47], and also has a wide normal variation.

STATISTICAL EVALUATION OF METHODS

The accuracy of the three methods was demonstrated by recovery experiments. To each of two sera rendered hypochloremic by dialysis, were added known amounts of sodium chloride. Each specimen was then analyzed, in triplicate, by each of the three methods. For each method the results were averaged for each serum sample and compared as shown in Table II.

Reproducibility of each of the three methods was demonstrated by performing ten replicate analyses of the same serum sample by each method. From the values so obtained the coefficient of variation[22, 69] for each of the three methods was calculated as follows:

Argentimetric:	1.3 per cent
Chloranilate:	0.89 per cent
Mercurimetric:	0.81 per cent

These results indicate that the deviation between the results of replicate analyses is small, and that reproducibility is good. Good reproducibility is also shown by the confidence limits of the mean, which when calculated for the 95 per cent level, were: argentimetric 0.57, chloranilate 0.56, and mercurimetric 0.60.

Using the "Student "t" Test"[18], the results obtained with the chloranilate and mercurimetric methods were compared with those obtained with the argentimetric method in 253 separate

TABLE II

Specimen	Argentimetric Result mEq./L.	Argentimetric Recovery %	Chloranilate Result mEq./L.	Chloranilate Recovery %	Mercurimetric Result mEq./L.	Mercurimetric Recovery %
Serum #1 –	73.3	–––	74.7	–––	71.0	–––
+20 mEq./L.	94.3	105	93.7	95	92.3	107
+40 mEq./L.	112.7	99	116.0	104	110.0	98
Serum #2 –	83.3	–––	81.0	–––	82.0	–––
+20 mEq./L.	98.3	93	96.6	104	96.3	96
+40 mEq./L.	114.6	105	111.3	101	112.6	102

serum samples. The resulting "t" values were,

Chloranilate-Argentimetric:	1.68
Mercurimetric-Argentimetric	1.75

showing that there is no significant difference between the results obtained by the methods compared.

The following isometric three-dimensional projections of the analytical data also demonstrate good agreement between the methods. Figure 4 shows a plot of the results obtained by the chloranilate method against those obtained by the argentimetric method. Figure 5 shows mercurimetric results plotted against argentimetric values.

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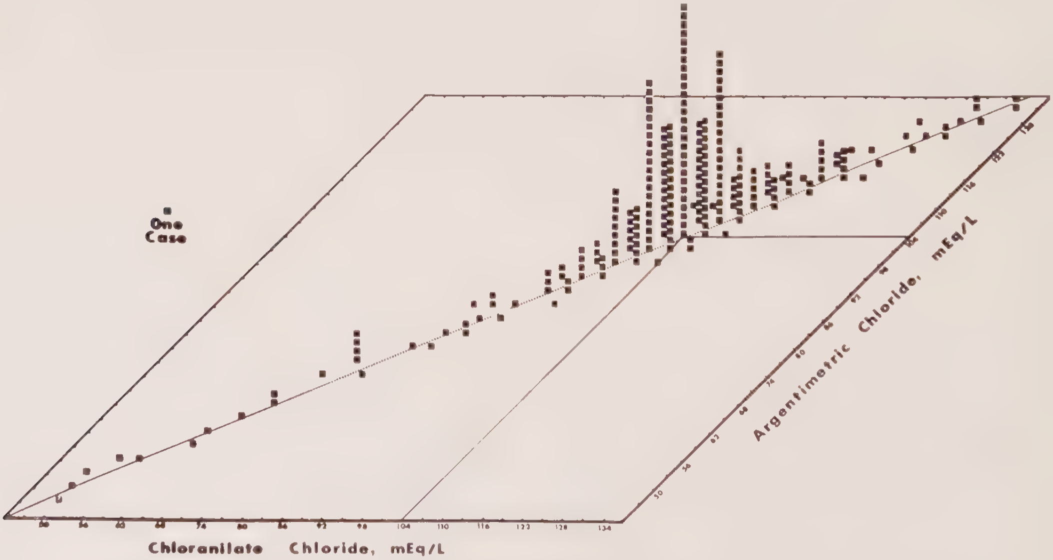

Fig. 4. Isometric, three-dimensional projection of results of chloride analysis on 253 serum samples by chloranilate technic plotted against results on same specimens by argentimetric technic. One black square represents one case. Diagonal dotted line represents theoretical agreement. Orthagonal grid lines (104 mEq/L) represent mean.

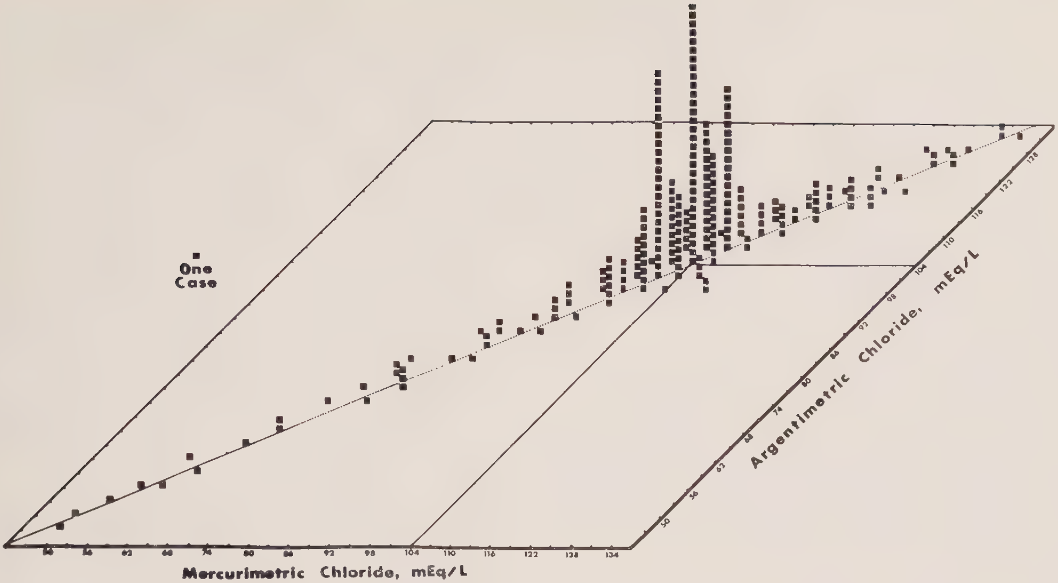

Fig. 5. Isometric, three-dimensional projection of results of chloride analysis on 253 serum samples by mercurimetric technic plotted against results on same specimens by argentimetric technic. One black square represents one case. Diagonal dotted line represents theortical agreement. Orthagonal grid lines (104 mEq/L) represent mean.

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The Measurement of Inorganic Sulfate in Biological Fluids

THOMAS E. WHEELER, Ph.D., J. THEODORE BRIERRE, JR., M.D. and LARRY G. DICKSON, M.D.

A. General Considerations

This chapter is concerned with the chemical technics for the determination of inorganic sulfate in serum and urine. The term *inorganic sulfate* refers to that fraction of the total sulfate in biological fluids which is available in an essentially protein-free medium, to be precipitated as an insoluble salt.

In 1914, Gauvin[1] and Rosenheim[2], working independently, developed technics which employed benzidine as a precipitating reagent for urine sulfate. The precipitated benzidine sulfate salt was then titrated in a boiling solution with standard base.

The first record of sulfate determination of blood was reported by DeBoer[3], in 1917, who used horses as subjects. His method required a specially calibrated centrifuge tube for the estimation of the volume of precipitated barium sulfate. In 1921, Denis[4], employing a turbidimetric procedure, determined the concentration of *inorganic sulfate* of human blood for the first time. He deproteinized 5 ml. of oxalated blood or plasma with mercuric ion and formed a suspension of barium sulfate by the addition of barium chloride to the filtrate. The formation of a colloid was facilitated by the use of ammonium nitrate. In 1928, Reed and Davis[5], using essentially this same method, determined the distribution of *inorganic sulfate* in a variety of bloods of different species including humans. It was demonstrated that the *inorganic sulfate* was concentrated primarily in the plasma. The results of their study of humans are shown in Table 1.

The volumetric method for titrating the benzidine sulfate precipitate from urine was modified by Fiske[6] to a more exacting technique. In 1923, this method for urine sulfate was first applied to a trichloroacetic acid filtrate of blood by White[7]. The filtrate was preliminarily dried and ashed and then analyzed by the Fiske benzidine method.

The use of benzidine as a colorimetric reagent for serum sulfate was proposed by Yoshimatsu[8]. In this procedure, the sulfate is precipitated with the benzidine reagent and the precipitate washed and redissolved. The benzidine is oxidized by iodine to yield a brown color, the intensity of which is interpreted in terms of sulfate concentraton. This method was modified by Hubbard[9], who employed trichloroacetic acid as a deproteinizing reagent and was able to obtain a sensitivity which was adequate for 2.0 ml. of serum. A dilute solution of benzidine was used as a standard. Color formation was brought about by oxidation of benzidine with hydrogen peroxide and ferric chloride. Hubbard also investigated the influence of phosphates and sodium chloride on the accuracy of his procedure and carried out recovery studies using potassium sulfate as a standard.

TABLE I AVERAGE RESULTS ON THE INORGANIC SULFATE OF BLOOD AND PLASMA[5]

(Expressed as mg. of S per 100 ml.)

	MAXIMUM	MINIMUM	MEAN
Human Whole Blood.	0.65	0.28	0.45
Human Plasma.	1.12	0.50	0.87

By the year 1931, representing a ten year span since the first method for serum sulfate by Denis had appeared, a total of six procedures which differed basically had been published and expanded by numerous modifications of each one. At this time, Cope[10] and Cuthbertson[11], working independently, evaluated and criticized the available methods. Both of these investigators proposed alterations in the benzidine procedure. Cope employed a micro-titration as an end-point in the procedure of Hubbard in lieu of an estimated color intensity. Cuthbertson diazotized the precipitated benzidine, followed by a coupling with thymol to produce an intense red color. Cuthbertson confirmed Reed's and Denis' observation that the inorganic sulfate was almost exclusively extracellular.

It is obvious that at the time of Cope's and Cuthbertson's reviews, benzidine was almost universally accepted as the reagent of choice for the precipitation of inorganic sulfate. However, the most desirable method of determining the benzidine was quite controversial. The necessity of standardization in this respect was recognized by Hoffman[12], who pointed out the discrepancies in the results which had been published by different investigators. Hoffman demonstrated that the conditions of sulfate precipitation were very critical. He observed that the acidity of the deproteinized serum filtrate will result in a loss of benzidine reagent if it is too strong, and the coprecipitation of phosphate if it is too weak. Hoffman developed a tedious procedure which was specifically designed to eliminate the fallacies existing in the other available methods. He used a permanganate titration of the precipitated benzidine as an end-point in his method. Unfortunately, Hoffman's procedure was too demanding in time and technique to enjoy an appreciable popularity, and the next year, Letonoff and Reinhold[13] developed a method which is, with some minor modifications, widely used today. This is a convenient colorimetric procedure based on the color producing reaction between benzidine sulfate and sodium beta napthoquinone-4-sulfonate. Phosphates and proteins are removed preliminary to analysis by the use of uranium acetate. Letonoff and Reinhold found that the *inorganic sulfate* of normal human serum determined by this method averages 1.04 mg. per 100 ml. The lowest value was 0.95 mg., the highest 1.16 mg. per 100 ml. Additional sulfate is liberated by hydrolysis when serum is treated with trichloroacetic acid for removal of proteins.

Kleeman[14] studied the spectral characteristics of the benzidine/beta napthoquinone-4-sulfonate derivative and adapted the procedure to a spectrophotometric technique. Kleeman also used an overnight period of precipitation for the benzidine sulfate.

Nephelometry, when carefully carried out, appears to be a valid technique for determining the concentration of *inorganic sulfate* in serum and urine. Nalefski[15] has developed such a procedure which employs the uranium acetate reagent of Letonoff and Reinhold as a precipitating reagent for proteins and phosphate. The procedure is quite similar to that proposed by Denis. The final suspensions of barium sulfate are read on a nephelometer. With this method Nalefski observed a normal range for the sulfate content of human serum to be 0.5 to 1.2 mg./100 ml.

The chloranilate method should be mentioned as potentially important in this field. The use of chloranilic acid in the colorimetric determination of serum calcium assumed a limited popularity a few years ago. The method lacked sensitivity and is not used extensively. In the procedure for inorganic serum sulfate, the insoluble barium chloranilate is used to precipitate barium sulfate. The reaction liberates an equivalent amount of soluble chloranilic acid which is then determined colorimetrically and the results interpreted in terms of sulfate. However, as in the calcium procedure, chloranilic acid is the colored compound measured, and the same limitations which were imposed on the calcium procedure must be considered here. The basic method is simple and rapid and has been used by Lloyd[16] for the determination of sulfate ions liberated from a substrate by incubation with sulfatases. With more work, the method may be appropriate for use with serum and urine.

The importance of establishing the normal range for the inorganic sulfate of serum for any specific method is obvious from the data in TABLE II. The wide variation of results which exists among different methods and different laboratories is shown in TABLE II. The analytic principles for the estimation of *inorganic sulfate* in biological fluids are summarized in Table III.

TABLE II CONCENTRATION OF NORMAL SERUM INORGANIC SULFATE AS DETERMINED BY VARIOUS INVESTIGATORS[15]

AUTHOR	PRECIPITATING AGENT	METHOD	mg. S/100 ml.
Denis	Barium	Turbidimetric	0.5-1.0
Kahn and Postmontier	Barium	Volumetric	1.0-3.0
Loeb and Benedict	Barium	Gravimetric	0.7-1.6
Wakefield	Benzidine	Colorimetric	0.2-0.9
Cuthbertson and Tompsett	Benzidine	Colorimetric	0.1-0.5
Wakefield, Power, and Keith	Benzidine	Oxidimetric	0.8-1.7
Macy	Benzidine	Oxidimetric	1.1-1.9
Hoffman and Cardon	Benzidine	Oxidimetric	0.3-1.1
Letonoff and Reinhold	Benzidine	Colorimetric	0.95-1.16
Nalefski and Takano	Barium	Nephelometric	0.5-1.2

TABLE III. ANALYTICAL PRINCIPLES FOR THE ESTIMATION OF INORGANIC SULFATE

	REAGENT	END POINT OF METHOD
Inorganic Sulfate Serum and Urine	+ barium	gravimetric
	+ barium	turbidimetric
	+ benzidine + H_2O_2 and $FeCl_3$	colorimetric
	+ benzidine + standard base	volumetric
	+ benzidine + $KMnO_4$	volumetric
	+ benzidine + I_2 + KI	colorimetric
	+ benzidine + sodium beta napthoquinone-4-sulfonate	colorimetric

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B. Benzidine Method for the Measurement of Inorganic Sulfate

INTRODUCTION

The inorganic sulfate concentration of serum or urine is routinely determined by one of several procedures employing benzidine as a precipitating agent. Benzidine demonstrates two properties which account for its frequent use in this role. First, it forms a highly insoluble salt with sulfate in acid solution which establishes specificity. Second, the benzidine may be redissolved and the sulfate titrated or color developed with the benzidine for analytical results. Hubbard's original method employing benzidine[1] subsequently developed color with ferric chloride and hydrogen peroxide. Unfortunately, the resulting color was weak and the method was relatively insensitive. Letonoff and Reinhold[2] modified the procedure and employed beta naphthoquinone-4-sulfonate to develop a red product with the amine group of the benzidine. These workers also employed uranyl acetate as a precipitating reagent for protein and simultaneously eliminated the interference by phosphate. Dodgson and Spencer[3] experienced significant loss of the benzidine in the precipitation and washing steps preceding color development. Further study of this problem, as well as the spectral characteristics of the beta naphthoquinone-4-sulfonate/benzidine derivative, were carried out by Kleeman, Taborsky and Epstein[4]. The method described here combines technics from both the Kleeman *et al.* and Letonoff-Reinhold methods.

REAGENTS

1. *Benzidine Solution.* One gram of benzidine is dissolved in 100 ml. of acetone and filtered. The solution is stored in a brown bottle in a refrigerator and discarded when it develops appreciable color.

2. *Color Reagent.* Pure sodium beta naphthoquinone-4-sulfonate (0.15 gm.) is dissolved in 100 ml. of distilled water. The solution is stable for approximately two weeks when stored in a refrigerator.

3. *Acetone.* Reagent grade.

4. *Sodium Hydroxide-sodium Borate Solution.* One gram of powdered sodium borate is dissolved in 100 ml. of 0.1 N sodium hydroxide.

5. *Uranyl Acetate Solution.* Eight tenths (0.8) gram of uranyl acetate are dissolved in 200 ml. of distilled water.

6. *Benzidine Standard.* Benzidine hydrochloride which is to be used for standardization is purified by dissolving 5 gm. in 200 ml. of 5 per cent hydrochloric acid and warming to approximately 50° C. The solution is filtered and 20 ml. of concentrated hydrochloric acid are added to the filtrate with continuous stirring. The solution is cooled in ice water for approximately thirty minutes and the crystals are collected on a Buchner funnel. The crystals are washed with cold dilute hydrochloric acid (15 per cent V/V) and dried under vacuum. The crystals are then washed with two 25-ml. portions of cold ethyl alcohol and four portions of ether. The ether

is removed by vacuum, and the dry crystals are transferred to a brown bottle.

7. *Stock Standard.* This solution is prepared by dissolving 0.1606 gm. of benzidine hydrochloride in 50 ml. of distilled water, which is contained in a 200-ml. volumetric flask. Solution is carried out at 50° C. The flask is cooled to room temperature, diluted to the mark, and stored in the cold.

8. *Working Standard.* Five milliliters of the stock solution of benzidine hydrochloride are diluted to 150 ml. with distilled water. The concentration of benzidine per milliliter of the dilute working standard is equivalent to 10 μg. of sulfate or 3.33 μg. of sulfur. The working standard should be stored in a refrigerator.

PROCEDURE

1. Six ml. of uranyl acetate solution are pipetted into a 15-ml. centrifuge tube.

2. Two ml. of non-hemolyzed serum are transferred slowly to the tube. The tube is covered with a piece of parafilm and mixed by inverting the tube four times.

3. The tube is centrifuged for ten minutes. Four ml. of the clear centrifugate are transferred to a second centrifuge tube.

4. To this second tube is added 1 ml. of glacial acetic acid, followed by 9 ml. of the benzidine solution. The tube is stoppered and placed in an ice water bath for two hours.

5. The tube is removed from the bath, and while still cold, centrifuged for fifteen minutes at 3000 r.p.m.

6. The supernatant is decanted. The tube is permitted to drain in an inverted position for three minutes.

7. Fourteen ml. of acetone are added to the tube, and the precipitate is resuspended with the aid of a stirring rod.

8. The tube is centrifuged as before, and the acetone is removed by decanting. The tube is inverted and allowed to drain for five minutes. At the end of this period, the mouth of the tube is wiped dry with a piece of filter paper.

9. One ml. of borate solution is added to the tube, and it is placed in a water bath, at 60°C with occasional gentle shaking until the contents are dissolved.

10 The tube is cooled to room temperature, and 10 ml. of water and one ml. of the beta naphthoquinone-4-sulfonate are added. The tube is allowed to stand for five minutes. Two ml. of acetone are added.

11. The tubes are mixed, centrifuged and the supernatnat fluid is decanted into cuvets for the Coleman Junior spectrophotometer or other suitable instrument. The color intensity is determined at a wavelength of 490 millimicrons, and the concentration is interpreted from a calibration curve.

PREPARATION OF A STANDARD CURVE

The method is standardized according to Letonoff and Reinhold[2] and adapted to the Coleman Junior spectrophotometer as described by Kleeman, Taborsky, and Epstein[4].

The standard curve is prepared by pipetting 0, 1, 2, 3, 4, 5, 6, and 7 ml. of the working standard, respectively, into each of eight centrifuge tubes. These standards are equivalent to 0, 10, 20, 30, 40, 50, 60, and 70 μg of sulfate, or one-third this equivalent of sulfur. The volume in each tube is adjusted to 10 ml. with distilled water. The sodium borate, color reagent, and acetone are added to each tube as described for the serum filtrate.

CALCULATIONS

The volume of the filtrate in the final step represents 1 ml. of serum. Therefore, the micrograms interpreted from the calibration curve multiplied by $\frac{100}{1000}$ (= 0.1) will express the result in milligrams of sulfur per 100 ml. of serum.

URINE

The inorganic sulfate concentration in urine is usually much higher than that of serum. The concentration will vary with many factors, including volume excreted per unit of time. Prior to analysis, the urine volume should be diluted to yield a concentration of sulfate sulfur between 3.5 and 16 μg per ml. of diluted urine. Two milliliters of the diluted specimen are treated exactly as serum in the method.

DISCUSSION

The results of the analyses are usually expressed in milligrams of sulfate sulfur per 100 ml. of serum. However, the concentration of sulfate will be used occasionally and may lead to some confusion if the reader is not aware of the use of the two systems. The sulfur content of sulfate closely approximates 33 per cent, and a rapid conversion from one system to the other may be made by appropriate employment of the factor 3.

There is confusion in the literature regarding a normal range for serum sulfate. The value of 1.1 mg. per 100 ml. of serum is generally accepted as an upper normal for human serum. However, it is imperative that each laboratory establish its own range for this anion.

The calibration curve is subject to occasional change, and its reproducibility should be established by incorporating one or two standards with each series of analyses. The curve will be observed to deviate from linearity in the range of 60 μg. of sulfate. Those specimens which fall in this range of higher concentration should be redetermined following a dilution of the serum filtrate.

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The Measurement of Inorganic Phosphorus in Serum

JOHN P. KALTENBACH, Ph.D., GERALD S. DEAN, M.D., and
GERALD G. HOFFMAN, M.D.

A. General Considerations

The quantitative estimation of an element such as phosphorus is usually, in itself, not too difficult. However, the presence in biological materials of proteins, lipids, carbohydrates, and their derivatives, makes the assay procedure more complex, because organic compounds tend to interefere with color development. In the case of serum inorganic phosphorus, the problem is not only the removal of interfering substances, but the development of a procedure that will be applicable to the clinical laboratory. This has led to a number of different methods and many modifications[18, 23].

In this chapter the problems involved in inorganic phosphorus determinations will be examined, and then some of the more common assay procedures will be discussed. It should be pointed out that the assay procedures actually determine the phosphate ion.

The principle of the most common procedure for estimating phosphate is the following: To the phosphate-containing sample, molybdate is added. This forms a phosphomolybdate complex. A suitable reducing agent is then added which will reduce this complex to give a blue color in an acid medium[25]. The intensity of the blue color is proportional to the amount of phosphate present.

The problems involved in this assay that have resulted in numerous modifications are: (a) The acid reagent for precipitating serum proteins must be strong enough to precipitate the proteins completely, though not so strong that it will interfere with color development. (b) Reducing agents are usually difficult compounds with which to work, as many are quite unstable; some may not be strong enough to reduce the phosphomolybdate complex; and a number will react at a narrow range of acid concentration. (c) Some methods are very sensitive to interfering substances and require the highest purity of reagents. (d) Color development proceeds slowly.

The reduction of the phosphomolybdic acid complex in an acid medium to yield a blue color was first described by Taylor and Miller in 1914[24]. It was later recognized, and this confirmed Feigl's original theory[18], that phosphates actually catalyze the rate at which molybdic acid is reduced[5, 6]. It has also been found that phosphorus is not the only element to complex with molybdic acid. Boltz and Mellon[7] described absorption spectra curves of reduced molybdic acid complexes of phosphorus, germanium, silicon, and arsenic. For this reason, the extraction of silicon from glassware, especially soft glass, can be a cause for abnormal values and colored blanks.

All of the commonly used procedures for phosphate analysis employ molybdate at an acid pH as the complexing agent. Most methods use ammonium molybdate, but Gomori[14] suggested sodium molybdate. The original Fiske-SubbaRow method used sulfuric acid to obtain the correct pH, and most subsequent modifications also use sulfuric acid. The use of a different acid, e.g., perchloric acid[16], acetate buffer[19], has usually been justified because of special experimental circumstances, e.g., analysis of labile phosphate esters, which are not present in the case of serum inorganic phosphorus.

The importance of maintaining certain narrow limits of acidity for maximum color development and reproducibility of results was shown by Briggs[8] and Fiske and SubbaRow[13]. Although different reducing agents may have slightly different requirements as to the final concentration of acidity, the working range, or the limits wherein optimum color is developed, is usually very narrow, e.g., 0.6 to 1.8 N[3]. Too much acid may reduce molybdate itself to a blue color[7] and result in artificially high values and colored blanks. Too little acid may result in low values.

Perhaps the most widely used method for the determination of phosphate is that of Fiske and SubbaRow[13]. For the reduction and development of the molybdenum blue color, Fiske and SubbaRow recommended 1-amino-2 naphthol-4-sulfonic acid (ANS) as a suitable reducing agent. The color produced slowly increases in intensity over a period of 120 minutes[14]. This is no disadvantage if a small number of samples are to be measured, as the color change is insignificant. If the number of samples would require twenty or thirty minutes to read in the colorimeter, then the standard would have to be interspaced and read between a short series of samples. Each series of samples would have to be calculated on the basis of the reading of the standard at that particular time. This calculation is possible because the color intensity of the standard will increase at the same rate as the samples.

Heat has been used to intensify and stabilize the color obtained with ANS[3, 4, 22]. Bartlett states that heating at 100° C for four or five minutes establishes maximum color and is stable for twenty-four hours. The color produced yields a peak at 830 mμ, which is 7.2 times higher than that observed by the conventional procedure and read at 660 mμ[3].

A number of different reducing agents have been proposed, not so much to overcome the lack of shelf stability which is common to many reducing agents, but to increase the stability of the final colored product. Gomori[14] recommended methyl-p-aminophenol sulfate (Elon, Eastman-Kodak) as being relatively inexpensive, stable in solution, and producing a stable color after thirty or forty minutes. The intensity of color produced approximates that of the Fiske-SubbaRow procedure. Methyl-p-aminophenol (Pictol, Mallinckrodt) produces maximum color in ten minutes, and remains stable through ninety minutes[15].

Ascorbic acid[9, 19, 20] with heat has been proposed as having greater sensitivity than the Fiske-SubbaRow method (molar absorbance of 25,000 compared to 4000)[20]. However, the ascorbic acid-molybdate-sulfuric acid mixture must be freshly mixed each day. The color remains stable for at least 120 minutes, following sixty minutes of heating at 37° C.[9].

A recent modification of the ascorbic acid reduction method has been the addition of an arsenite-citrate reagent to stabilize the reduced phosphomolybdic acid complex[1, 2]. The color is reported to be stable for twenty-four hours.

Optimum conditions for stannous chloride reduction of phosphomolybdate were described by Kuttner and Cohen[17]. The concentrated stock solution of stannous chloride is stable, but the dilute reagent must be prepared daily[14] or weekly[26]. The reagent is very sensitive to impurities and requires the maintenance of very narrow acid tolerance for optimal color development. The developed color is stable[14, 26].

An incisive investigation of phosphate analysis of Berenblum and Chain[5] resulted in an adaptation of the stannous chloride method that virtually eliminates interfering substances. The principle is that the phosphomolybdic acid complex is very soluble in organic solvents and is extracted into an organic solvent layer as soon as it is formed. Although this method may be a bit involved for routine analysis of serum inorganic phosphorus, it serves as an excellent method for the determination of tracer phosphate in research studies[12].

The spectrophotometric studies of Boltz and Mellon[7], using hydrazine sulfate as the reducing agent, demonstrated a marked absorption band at 830 mμ. These workers proposed the term "heteropoly blue" for systems forming the molybdiphosphoric acid complexes and having this absorption characteristic. The samples are heated for ten minutes in boiling water, and the color is stable for twelve hours.

Dryer and co-workers[10, 11] recommended N-phenyl-p-phenylenediamine hydrochloride (Semidine hydrochloride). Semidine is available in a suitable state of purity. Maximal color development is in ten minutes and remains constant for at least ninety minutes. Semidine, similar to ANS, should be protected from light and prepared freshly every three to four weeks. Although the color can be read at any point between 650 and 750 mμ, greatest sensitivity is obtained at 345 mμ.

Recently, Negrin[21] has proposed using hydriodic acid as the reducing agent. The color produced is stable for one hour, if strict adherence to pH is maintained. Sensitivity is claimed to be two and one-half times that obtained with ANS. This procedure has not had an opportunity to be evaluated by others.

Other reducing agents have been used, but they have not had sufficient advantages to warrant widespread use. Several methods do not use a reducing agent[18]. These methods have also not become popular, although the yellow phosphovandomolybdate complex has been described as one of three means of automated determination of serum phosphorus[1].

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B. Molybdate Method for the Measurement of Inorganic Phosphorus in Serum

INTRODUCTION

The method of phosphorus determination, according to Fiske and SubbaRow[1], with minor modifications, is still a recommended[2] and widely used procedure. Their incisive investigation also included a critical evaluation of methodology in phosphorus determinations. Not until relatively recently have new innovations been introduced or methodology re-evaluated e.g.,[3, 4, 5]. Although other methods may have certain advantages, the Fiske and SubbaRow method will be described because of its general acceptance and usage in the clinical laboratory.

PRINCIPLE

The colorimetric estimation of inorganic phosphorus is based upon the formation of a phosphomolybdate complex. A reducing agent is added, in this case aminonaphtholsulfonic acid, which reduces this complex to "molybdenum blue". The intensity of the blue color is proportional to the amount of phosphorus present.

REAGENTS

1. *Trichloroacetic Acid, 10 per cent.* This may be prepared from a stable concentrated stock solution (100 grams crystaline trichloroacetic acid (TCA) made to 100 ml. with distilled water) by diluting the concentrated stock standard 1:10 with distilled water. The dilute TCA should be kept refrigerated and prepared freshly every two to four weeks.

2. *Sulfuric Acid, 10 N.* Four hundred and fifty ml. of concentrated sulfuric acid (sp. gr. 1.84) are added slowly to 1300 ml. of distilled water in a Pyrex container. The solution is allowed to cool and is stored in a glass-stoppered Pyrex bottle. The importance of maintaining the critical concentration of acid for maximum color development requires that the sulfuric acid be titrated[1, 3]. This reagent is shelf stable indefinitely. It is recommended that a small working bottle of sulfuric acid reagent be filled from the stock solution to prevent contamination of the stock.

3. *Sodium Bisulfite, 15 per cent.* Thirty grams of sodium bisulfite are dissolved and diluted to 200 ml. with distilled water. This reagent should be freshly prepared and should not be cloudy.

4. *Sodium Sulfite, 20 per cent.* Twenty grams of sodium sulfite are dissolved and diluted to 100 ml. with distilled water. Because of the ease of preparation of this solution, storing is not recommended.

5. *Molybdate Reagent (Molybdate II).* Twenty-five grams of ammonium molybdate are dissolved in approximately 200 ml. of distilled water. Three hundred ml. of 10 N sulfuric acid are placed in a one-liter volumetric flask. The molybdate solution is quantitatively transferred into the flask containing the sulfuric acid and made to the one-liter mark with water. This reagent is shelf stable.

6. *Aminonaphtholsulfonic Acid*

Reagent. Five tenths (0.50 gram) of a gram of 1-amino-2-naphthol-4-sulfonic acid are added to 195 ml. of 15 per cent sodium bisulfite. Five ml. of 20 per cent sodium sulfite is then also added. The mixture is shaken until dissolved. If solution is not complete, one ml. quantities of sodium sulfite solution are added with shaking until solution is complete. This solution is stable for about one month, if refrigerated and exposure to air and light kept at a minimum.

7. *Phosphorus Standard Stock Solution (1 mg. Phosphorus per ml.).* Exactly 0.4394 gram of anhydrous potassium dihydrogen phosphate (KH_2PO_4) is dissolved in 100 ml. of distilled water containing one ml. of 10N sulfuric acid. A few drops of chloroform may be added to prevent growth of mold. This solution is shelf stable.

8. *Phosphorus Standard Working Solution (0.005 mg. Phosphorus per ml.).* One ml. of stock standard is diluted to 200 ml. with 10 per cent trichloroacetic acid.

PROCEDURE

1. (a) *Sample.* To nine ml. of 10 per cent trichloroacetic acid one ml. of serum or plasma is added. The serum is added slowly and with constant mixing. The mixture is centrifuged or filtered, and 5 ml. of the filtrate are transferred to a photometer cuvet.

(b) *Reagent Blank.* Five ml. of 10 per cent trichloroacetic acid are measured into a photometer cuvet.

(c) *Phosphorus Standard.* Five ml. of the working standard are measured into a photometer cuvet

2. One ml. of the molybdate reagent is added to each cuvet and mixed.

3. Aminonaphtholsulfonic acid, 0.4 ml., is then added to each cuvet and mixed.

4. To each cuvet are added 3.6 ml of distilled water to make the final volume 10 ml.

5. Color development is allowed to proceed for ten minutes before reading at 660 mμ against the reagent blank. The reagent blank should be colorless.

CALIBRATION CURVE

1. To a series of 10 ml. volumetric flasks are added 0, 1, 3, and 5 ml. of the phosphorus working standard

2. To each flask is then added 5, 4, 2, and 0 ml. of 10 per cent trichloroacetic acid.

3. Proceed as in *steps 2 to 5 of Procedure.*

DISCUSSION

The blue color of this procedure can be read over a wide range of wavelengths because of the flat nature of the absorption curve, peak absorbance being at 820 mμ[7]. The optimum wavelength thus depends upon convenience and sensitivity of the instrument used. The suggested wavelength of 660 mμ is suitable for such commonly employed instruments as the Coleman Jr. Colorimeter.

The concentration of trichloroacetic acid is another variable encountered in the literature. Under the conditions described in *Procedure,* using one ml. of serum, 5 to 10 per cent trichloroacetic acid will serve to precipitate the proteins. Greater concentrations of acid may interfere with color development.

Color development continues slowly, but increases in intensity for as long as 120 minutes[3]. This is not a serious disadvantage. If a small number of samples to be read is so great that the time involved would require twenty to thirty minutes, then the standard would have to be periodically read between a short series of samples. Each series of samples would have to be calculated on the reading of the standard at that particular time. This type of calculation is possible because the color intensity of the standard will increase at the same rate as the samples.

SOURCE OF ERROR

1. It should be noted that the amount of phosphorus actually measured in the serum sample is usually less than 25 micrograms, and the dilution factor is 200. Therefore, it is important that all reagents, including the distilled water, are of the highest purity, and all glassware scrupulously clean.

2. Care must be exercised to avoid detergents containing phosphates (a common cause of colored "blanks").

3. Hemolyzed serum should not be used because phosphatases in the red blood cells may have liberated inorganic phosphate from the organic phosphates present. For the same reason, separation of serum should be done as soon as possible.

4. Plasma from oxalated blood may be used, although excessive amounts of oxalate must be avoided, as this tends to inhibit color formation.

5. Heparin may contain phosphate as a contaminant. However, this is usually too low to interfere. If in doubt, a heparin blank can be run.

6. Fasting samples of blood are recommended, as variation of serum phosphorus can occur during metabolism of carbohydrates[3, 6].

7. The working standard should always have approximately the same optical density value. This can serve as a check for pipeting errors and colorimeter fluctuation.

NORMAL VALUES[8]

Adults: 3.2 to 4.3 mg. per 100 ml.
(1.6 to 2.7 mEq. per L.

Infants: 4 to 7 mg. per 100 ml.

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The Measurement of Organic Acids in Serum

A. General Considerations

ALFRED H. FREE, Ph.D., and HELEN M. FREE, B.S.

The organic acids of serum represent a myriad of substances with a large variety of origins and functions. A recent review by Nordmann and Nordmann[13] mentioned over one hundred different organic acids which may be found in blood or serum.

In either health or disease, there is little effort made to measure directly or to identify the total organic acids of the serum. Changes in the total equivalent amount of organic acids can be recognized by the difference between the total cations, or total base of the serum, and the anions which are measured. In certain instances, which will be discussed, identification or measurement of certain organic acids can provide important information which is useful in diagnosing or following the course of treatment of specific diseases.

Methods will be described in this chapter for quantitative determination of serum lactic acid and ketone bodies and for gas chromatographic fractionation of serum fatty acids. Although a number of other organic acids have diagnostic significance, methods for measuring these will not be described, since these acids do not contribute significantly to the electrolyte composition of the serum. Included in this group are pyruvic acid, alpha-ketoglutaric acid, citric acid, formic acid, acetic acid, propionic acid, all of the amino acids, phenylpyruvic acid, ascorbic acid, homogentisic acid, vanilmandelic acid, hippuric acid, oxalic acid, and many others.

The normal value for the total organic acids of serum is approximately 6 mEq. per L. There is normally about 1 mEq. of lactate per liter present, and the remaining 5 mEq. arise from very small contributions of a very large number of organic acids. Ordinarily, none of these constitute as much as 1 mEq. per L.

In diabetic ketosis, the ketone acids may increase from less than 1 mEq. per L. to more than 20 mEq. per L.[6]. In salicylate intoxication, the salicylate may be as much as 5 mEq. per L. whereas there is normally no salicylate in serum. In lactic acidosis, massive elevations of lactate occur, and in severe cases, as much as 25 mEq. per L. may be present[9]. Seligson and associates[17] have reported increases in organic acid from a normal 6 mEq. per L. to as much as 26 mEq. per L. in severe renal diseases. In acute methyl alcohol intoxication, quantities of formic acid as great as 15 mEq per L. have been noted[12].

RÉSUMÉ OF CLINICAL INTERPRETATION

Massive changes in the organic acids component of the serum and interstitial fluid may occur in ketoacidosis. The accumulation of acetoacetic acid and beta-hydroxybutyric acid gives rise to the severe acidosis which is encountered in this condition. The mechanisms of the metabolic disturbance have been recently reviewed by Shreve[18]. Duncan[4] and many others have emphasized the diagnostic utility of a simplified test for serum ketone bodies in ketoacidosis. It appears that acetoacetate is the principle nitroprusside-reacting substance which appears in the urine and in the serum in ketosis[7]. The utility of such a test in following the course of treatment of diabetic acidosis is also recognized.

Lactic acid may accumulate in the serum in a condition which has been called "lactic acidosis"[9]. Modest changes in lactic acid content of serum are encountered following vigorous muscular exercise[8], but much more extensive changes are noted in specific disease states. Severe anoxia and shock have a profound influence in elevating serum lactic acid. In such cases, it is likely that the increase in blood lactate in shock is due to tissue under-perfusion with a resultant increase in anaerobic metabolism. Several investigators[2, 9, 14] have pointed out that extreme elevations of lactic acid are encountered in irreversible shock, and that measurement of serum lactate in such conditions can provide important information. Appreciation of the fact that lactate accumulation occurs in certain disorders poses a question of the utility of sodium lactate solution as a parenteral fluid for treating acidosis[15]. Recently, severe lactic acidosis has been described in a limited number of diabetic patients receiving the drug phenformin[1, 19]. These patients were seriously ill, and whether the phenformin aggravated the development of lactic acidosis is not known.

The metabolism of pyruvic acid is intimately associated with the metabolism of lactic acid. Pyruvic acid, as such, never accumulates in the blood in sufficient quantities to cause a disarrangement of the acid-base equilibrium. The measurement of pyruvic acid in instances where serum lactic acid is increased can provide important information relating to the nature of the disordered lactate metabolism[8].

Salicylate intoxication occurs with great frequency, either as a result of accidental ingestion of aspirin tablets by children, or as a result of excessive ingestion by older individuals. A simplified test for salicylate in urine is an extremely useful procedure in any instance of suspected salicylate intoxication. If the urine is negative, this clearly reduces the possibility of salicylate intoxication. With a positive urine test in suspected cases, it is necessary to measure serum salicylate[10].

In methyl alcohol intoxication, a severe acidosis is encountered. This is due to the accumulation of formic acid[12]. The acidosis may further be aggravated in the later phases by appearance of ketone bodies of starvation origin. In contrast to methyl alcohol intoxication, with ethyl alcohol intoxication only very mild changes in the acid-base equilibrium occur[15]. A small increase in unidentified organic acids of serum is seen in either experimental animals or humans.

It is well recognized that in renal failure, a severe acidosis occurs. A number of factors contribute in varying degree to this acidosis. Seligson and associates[17] reported marked elevations in unidentified organic acids in serum of certain patients with renal disease.

Alpha-ketoglutaric acid is one of the intermediaries in carbohydrate metabolism. In certain liver disorders, this acid may be markedly increased in serum[3]. However, the amount of accumulated acid is not of such a magnitude to alter directly the acid-base equilibrium.

In hyperoxaluria an increase of oxalic acid in the urine occurs, but this is not associated with a significant alteration of oxalic acid in the serum[11].

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B. Measurement of Lactic Acid in Serum and Blood (Barker Method[1, 2])

ALFRED H. FREE, Ph.D., and HELEN M. FREE, B.S.

PRINCIPLE

Lactic acid in a protein-free filtrate of blood or serum is quantitatively oxidized to acetaldehyde with heat and concentrated sulfuric acid. Acetaldehyde is then condensed with *p*-hydroxydiphenyl in the presence of copper to produce a purple color which may be measured by use of a spectrophotometer.

REAGENTS

1. *Protein precipitant* (preferably Ba $(OH)_2 - ZnSO_4$ combination)
2. *Copper sulfate solutions* (20 per cent and 4 per cent $CuSO_4 \cdot 5H_2O$)
3. *Powdered calcium hydroxide*
4. *Sulfuric acid,* concentrated (pretested to ensure that color is formed with standard lactate)
5. *p-Hydroxydiphenyl* (1.5 per cent solution in 0.5 per cent NaOH, warmed slightly if necessary, to dissolve *p*-hydroxydiphenyl). This solution is stored in a dropping bottle whch delivers 0.05 ml. per drop.
6. *Lactate Standard* (Lithium lactate equivalent to 1 mg. lactic acid per ml.). To 50 ml. of water is added a quantity of 106.6 mg. of lithium lactate. Following solution of the salt, 20 ml. of approximately 1 N sulfuric acid are added and the solution is made to 100 ml. with water. Standards of 2 to 10 μg per ml. are made by dilution from this stock solution every few days. All standards are refrigerated.

PROCEDURE

1. One ml. of a 1:10 protein-free filtrate is mixed with 1 ml. of 20 per cent copper sulfate and 8 ml. of water in a centrifuge tube by inversion, using Parafilm to cover the mouth of the tube.
2. One gram powdered calcium hydroxide is added, and the mixture is shaken vigorously. If the mixture is not bright blue, more calcium hydroxide should be added.
3. The tube is allowed to remain at room temperature for thirty minutes, with occasional shaking, and then centrifuged.
4. One ml. of the copper-calcium supernatant is mixed with one drop of 4 per cent copper sulfate, and the tube is placed in a water-ice bath. Six ml. of chilled sulfuric acid are added.
5. The tube is heated in a boiling water bath for five minutes, then placed in cold water to cool to below room temperature.
6. Two drops of *p*-hydroxydiphenyl are placed directly on the solution in the tube, and the tube is shaken thoroughly.
7. The tube is placed in water at 30° C. for thirty minutes, and at least once during this time, the tube is shaken to disperse the reagent throughout the mixture.

8. Excess reagent is destroyed by heating the tube in a boiling water bath for one and one-half minutes.

9. After cooling, the tube contents are read within one hour at 570 mμ against sulfuric acid.

10. Calculations are made by the use of a calibration curve and appropriate dilution factors.

SOURCES OF ERROR

1. In order to avoid contamination of the sample with lactate of sweat or other body fluids, one should not touch the tips of pipets or the lips of the tubes.

2. Surface particles should be avoided when pipetting aliquots from the calcium-copper supernatant.

3. Tubes must be well-cooled before addition of *p*-hydroxydiphenyl, since the reagent disappears in sulfuric acid above 35° C. The reagent precipitates on contact with the acid, so it must be added directly to the solution and not allowed to run down the side of the tube.

4. The last heating period must be no longer than two minutes to avoid changes in the spectral characteristics.

5. Protein-free filtrate should be obtained by centrifugation to avoid contaminating material which might possibly be leached from filter paper.

DISCUSSION

The reaction for lactate is one which requires care in order to achieve satisfactory results. When properly carried out, the reaction is relatively specific and is not influenced to any significant degree by other constituents of the blood. Recently an enzymatic procedure for measuring serum lactate, employing lactic dehydrogenase enzyme has had an increasing popularity[3, 4]. This method involves measurements in the ultraviolet region of the spectrum and, accordingly, requires a UV spectrophotometer. A method involving a colorimetric (500 mμ) enzymatic procedure has also been described[5].

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C. Measurement of Ketone Bodies in Blood and Serum

SIDNEY S. CHERNICK, Ph.D.

INTRODUCTION

The quantitative estimation of ketone bodies (acetoacetate (AAA), β-hydroxybutyrate (β-HB) and acetone) requires the conversion of the four carbon acids to acetone and the subsequent determination of the acetone. Free acetone is less than 10 per cent of the total ketone bodies in body fluids. In the presence of acid, AAA undergoes thermal decarboxylation quantitatively producing acetone. With a satisfactory oxidizing agent, dichromate, β-HB is converted to acetone in yields approximating 90 per cent. However, such drastic treatment as heating with sulfuric acid and dilute dichromate will also oxidize other substances to aldehydes and ketones. For this reason, blood, plasma, and serum samples are deproteinized, and urines, and sometimes other body fluids, are treated to remove glucose and similar substances.

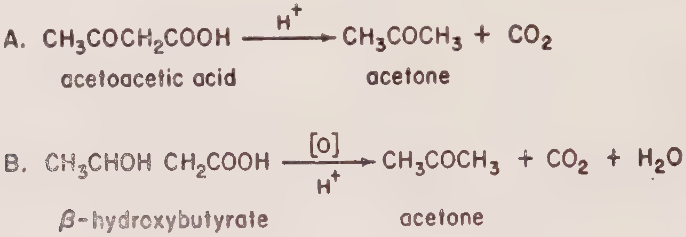
Deproteinization with barium hydroxide and zinc sulfate by the procedures widely employed for glucose determination[7] is adequate for most methods for the measurement of ketone bodies. For the removal of glucose from urine, the method employing calcium hydroxide and copper sulfate gives good results[6]. Pyruvate, lactate, and ethanol are not removed by these purification procedures; when present in large quantities, they may interfere with subsequent determinations of acetone.

The multiplicity of techniques for the quantitative determination of acetone is an indication of the lack of a good simple method. The two derivatives of acetone most commonly employed for its colorimetric measurement are those obtained with salicylaldehyde and 2:4 dinitrophenylhydrazine. Natelson[5] has presented procedures, employing 0.05 to 1.00 ml. of body fluid as the initial sample, that permit the use of several methods of acetone determination. Mayes and Robson[4] have modified the dinitrophenylhydrazine procedure to permit the measurement of total ketone bodies in 0.1 ml. of body fluid. The primary disadvantages of these methods are the distillation procedure and the length of time involved when many samples are to be assayed. Of the methods employing salicylaldehyde, that of Bessman and Anderson[2] has the advantages of speed and sensitivity. In the method of Bloom[3] distillation of acetone is carried out in modified Thunberg tubes with alkaline salicylaldehyde in the caps. This latter method permits the measurement of acetone in the range of 1 to 8 mcgm. per sample.

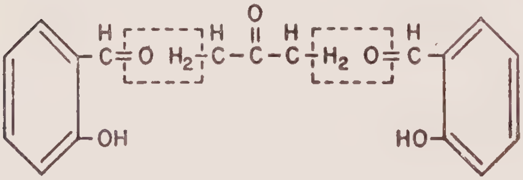
PRINCIPLE

The method of Behre and Benedict[1] has been modified and reduced to the micro scale by Bessman and Anderson[2]. The reactions employed in the conversion of the ketone bodies to acetone are:

1. Thermal decarboxylation of acetoacetic acid (A in Fig. 1).
2. Oxidation of betahydroxybutyric acid to acetoacetic acid by dichromate and decarboxylation (B in Fig. 1)


A. $CH_3COCH_2COOH \xrightarrow{H^+} CH_3COCH_3 + CO_2$
acetoacetic acid
acetone
B. $CH_3CHOH\ CH_2COOH \xrightarrow[H^+]{[O]} CH_3COCH_3 + CO_2 + H_2O$
β-hydroxybutyrate
acetone

In the presence of alkali, one molecule of acetone condenses with 2 of salicylaldehyde to form the red dye, dihydroxybenzalacetone (Fig. 2).



dihydroxybenzal-acetone

REAGENTS

1. *Deproteinization reagents* (Somogyi[7, 8]), 0.3 N Ba $(OH)_2$ and 5 per cent $ZnSO_4$.
2. *Sulfuric acid,* 13.6 N.
3. *Potassium dichromate,* 0.4 per cent (W/v).
4. *Salicylaldehyde*
5. *Potassium hydroxide,* saturated solution.
6. *Ethanol,* 50 per cent (v/v).
7. *Stock Standards.* Redistilled ethyl acetoacetate, 0.01 M and sodium betahydroxybutyrate, 0.01 M. These stock solutions are kept frozen.
8. *Working standards.* One ml. of each of the stock standards is diluted to 100 ml. with water, yielding solutions containing 0.1 mcM. per ml. (5.8 mcgm. acetone/ml.). These working standards are kept under refrigeration.

APPARATUS

1. *Aluminum-heating Block.** The heating block is adjusted so that only the lower two inches of an inserted tube are heated. An air stream or fan is used to cool the upper portion of an inserted tube so that its contents may be refluxed during the heating period.
2. *Reaction Tubes.* Culture tubes, 16 x 150 mm. with Teflon®-lined screw caps (Kimble No. 45066A) are satisfactory. Extra caps should be ordered (No. 45066C).
3. *Beckman Model B Spectrophotometer or Equivalent.* The wave length of choice is 500 mμ. When a spectrophotometer is employed, contaminants may be detected by calculating the ratio of the readings at 500 and 520 mμ.

PROCEDURE FOR BLOOD AND SERUM

Two tenths ml. of blood or serum is added to 1.8 ml. of water in a 12-ml.

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*Research Specialties Co., 200 S. Garrard Blvd., Richmond, Calif., Cat. No. 2125 with ¾" hole tube block or No. 2127 with hole blocks No. B-3.

conical centrifuge tube. One ml. of $Ba(OH)_2$ solution is added, and the contents are mixed. One ml. of $ZnSO_4$ is then added, and the tube is capped with parafilm or aluminum foil and shaken. The tube is centrifuged at 2,500 rpm for five minutes, and 1 ml. aliquots of the supernatant solution are taken for analysis.

Acetoacetate. To 1-ml. aliquots in screw-capped tubes, 0.25 ml. of 13.6 N H_2SO_4 is added, and the tubes are tightly closed with Teflon®-lined caps. The tubes are placed in a heating block set for 130° for fifteen minutes. A stream of air is used to cool the upper part of the tubes. After the heating period, the tubes are cooled in water, and two drops of salicylaldehyde are added, followed immediately by 2 ml. of saturated KOH solution. The tubes are capped, shaken, and allowed to stand at room temperature for thirty minutes for color development. Two ml. of 50 per cent ethanol are allowed to flow down the side of each tube. The contents are mixed vigorously, and another 2 ml. of the ethanol solution are allowed to flow down the side of the tube. After five minutes the contents are gently mixed by tapping. The tubes are centrifuged slowly for five minutes, and the clear yellow-to-orange supernatants are decanted into cuvets. Optical densities (S_a) are determined at 500 mμ

Betahydroxybutyrate. One-ml. aliquots of protein-free filtrate are treated as described for acetoacetate measurement through the first heating of fifteen minutes. The tubes are cooled, and 0.25 ml. of 0.4 per cent $K_2Cr_2O_7$ is added to each tube. The tubes are replaced in the heating block for an additional thirty minutes, cooled, and the color is developed as previously described. The resultant color (S_b) represents the total ketone bodies.

A blank and an acetoacetate standard (A_1) are carried through the procedure as described for measurement of acetoacetate. A blank, an acetoacetate standard (A_2) and a betahydroxybutyrate standard (B_{std}) are carried through the procedure as described for betahydroxybutyrate. The blank samples are used to adjust the photometer to zero optical density.

CALCULATIONS

The concentration of acetoacetate is calculated in the conventional manner and expressed as mg. of acetone. The concentration of betahydroxybutyrate is calculated by subtracting the concentration of acetoacetate from that of total ketones. However, since some acetoacetate is destroyed during the second heating, a correction must be made for loss of acetoacetate in order to estimate the concentration of betahydroxybutyrate.

The following expression is employed for the calculation of betahydroxybutyrate (B_u):

$$B_u = S_b - \left(\frac{A_2}{A_1} \text{x } S_a\right)$$

then:

$$\text{Betahydroxybutyrate (mg. per 100 ml.)} = \frac{B_u}{B_{std}} \text{ x (std. in mg. acetone)}$$

$$\text{x aliquot factor, where the aliquot factor is usually } \frac{100}{0.2} \text{ x } \frac{4}{1}$$

MEASUREMENT OF URINE KETONES

When the urine gives a positive test for glucose, it is necessary to treat it with copper and lime to permit the action of the oxidizing reagent on hydroxybutyrate. The treatment described by Peters and Van Slyke[6] is satisfactory. A sample of urine (10 ml.) is diluted with four volumes of water, and two volumes of 20 per cent $CuSO_4$ solution are then added. Two volumes of 10 per cent $Ca\ (OH)_2$ suspension are added, and the solution is tested for alkalinity. If not alkaline, more $Ca\ (OH)_2$ is added. Finally, water is added to complete a 1:10 dilution. After standing at room temperature for one hour, the suspension is filtered through a fluted paper.

To determine the aliquot for analysis, the urine sample is tested with "Acetest" or "Ketostix." These tests detect only acetoacetate and acetone. The principal component in ketosuria is betahydroxybutyrate. Each laboratory should prepare its own dilution scale, based on these qualitative tests.

A typical dilution scale is as follows:

Urine Acetest Color	Dilution (Including Cu + Ca Treatment)
Negative	1:10
Trace (pink)	1:50
Violet	1:100
Purple	1:500-1:1,000

The determination of acetocetate and betahydroxybutyrate is the same as that employed for plasma and blood.

COMMENTS

When estimations of total ketones are desired, the standard of choice is betahydroxybutyrate, since it is the major component. Moreover, the degree of destruction of acetoacetate (10 to 15 per cent) and the oxidation of betahydroxybutyrate to acetone (85 to 95 per cent) approximately balance each other.

The tubes and the caps must be checked to ensure a tight seal. Caps may be reused but only after careful inspection. Tubes that are cracked or chipped must be discarded.

RANGE OF VALUES

In normal individuals, the amount of total ketone bodies in the blood is small, in the fed or postabsorptive states being equivalent to less than 1 mg. acetone per 100 ml. plasma. During starvation, values increase and may reach 20 to 30 mg. per 100 ml. In diabetes, values in excess of 50 mg. per 100 ml. are common.

Urinary excretion of ketone bodies tends to follow blood levels. In normal individuals, as much as 100 mg. per day may be excreted. In uncontrolled diabetes, the values may exceed 1 gram per day. The proportion of acetoacetate and betahydroxybutyrate in the urine does not appear to be fixed but tends to vary with the amount excreted. In general, increases in the level of ketone bodies in blood and urine tend to favor increases in the proportion of betahydroxybutyrate.

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D. Gas Chromatographic Method for Fractionation of Fatty Acids in Serum

ALFRED H. FREE, Ph.D., and HELEN M. FREE, B.S.

INTRODUCTION

The principles of gas chromatography were first described by Martin and Synge[13] over twenty years ago. However, only within the past few years has a relatively large number of instruments become available allowing the procedure to emerge as a useful analytical tool. A recent text has listed thirty-three manufacturers of gas chromatographs throughout the world, twenty of these companies being in the United States[3].

Much of the work in gas chromatography methodology has been done in fields unrelated to clinical chemistry. Examples are separation of the chemical compounds responsible for the odor of perfumes or the taste of alcoholic beverages, and the automatic monitoring of the cracking process in petroleum refineries. The gas chromatograph is well-suited to the separation and identification of small quantities of compounds in organic mixtures and, indeed, one of the first analytical applications of gas liquid chromatography was the separation of volatile fatty acids by James and Martin in 1951[7]. In the clinical field, gas chromatography is now being applied to a large variety of measurements, including fatty acids, long-chain aldehydes, steroids, blood gases, intermediates in the Krebs tricarboxylic acid cycle, carbohydrates from diose to tetrasaccharide, alkaloids, sympathomimetic amines, aromatic acids in urine, and a wide variety of miscellaneous compounds.

PRINCIPLE

Gas liquid chromatography (GLC), as applied to determination of serum fatty acids, is a form of partition chromatography. Mixtures of fatty acid esters are volatilized and swept into a stream of carrier gas, which transports the mixture through the column packed with an inert material impregnated with a liquid of low volatility, or through a capillary column coated with the liquid. At the end of the column, a detector system is used to measure the composition changes in the effluent gas by thermal conductivity or ionization, and these changes are transmitted to a recorder. Calculation is made from the resulting peaks to indicate what percentage of the total fatty acid sample is due to each single acid fraction. Single acid fraction peaks are identified by their retention time (or how fast they emerge from the column).

REAGENTS

1. *Esterification Reagents.* Esterification reagents necessary for conversion of serum fatty acids to their corresponding methyl esters, such as diazomethane or HC1 and methanol.
2. *Stationary Phase.* Packed column of celite 545 coated with succinate polyester of diethylene glycol or capillary column coated with Apiezon L.
3. *Moving Phase.* Carrier gas (argon, hydrogen, nitrogen, helium).

STANDARD SOLUTIONS

It is recommended that reference standards be used daily in gas chromatography experiments. Such mixtures of fatty acid methyl esters recommended by the National Heart Institute[6] and shown in Table I are now available commercially from the Applied Science Laboratories, P. O. Box 140, State College, Pennsylvania. Since each component is calculated as a percentage of the total fatty acid methyl esters, it is necessary to include an internal standard of known weight or volume if results are desired in terms of weight or volume of sample.

TABLE I. COMPOSITION OF NHI STANDARD FATTY ACID METHYL ESTER MIXTURES IN WEIGHT PER CENT

	MIXTURE					
ACID*	A	B	C	D	E	F
8:0			1.5		6.3	
10:0			3.0		9.1	
12:0			6.0		12.1	
14:0	25.0	4.0	12.0	11.8	23.3	2.5
16:0	10.0	40.0	19.4	23.6	49.2	4.2
16:1				6.9		
18:0	65.0	56.0	24.9	13.1		7.3
18:1				44.6		
20:0			33.2			13.6
22:0						25.4
24:0						47.0

*Carbon chain length: double bonds

SPECIAL APPARATUS

Figure 1 presents a stylized diagram of the gas chromatograph and recorder. The carrier gas, usually helium in the United States, flows past the sample injector where the mixture of methyl esters of fatty acids is introduced. The carrier gas (the moving phase) sweeps the sample into the column, which is packed or coated with a polyethylene glycol succinate or adipate or with Apiezon stopcock grease — the stationary phase — at a temperature of about 200° C. The individual methyl esters are partitioned between the gas and liquid phase, depending on the length of their carbon chains and the degree of saturation or unsaturation.

Usually the short-chain and the saturated fatty acid esters have the shortest retention times. In other words, they are more soluble in the gas than in the grease. As the effluent gas reaches the ionization detector, the organic molecules are ionized and the resulting change in electrical conductivity is automatically recorded. With the less

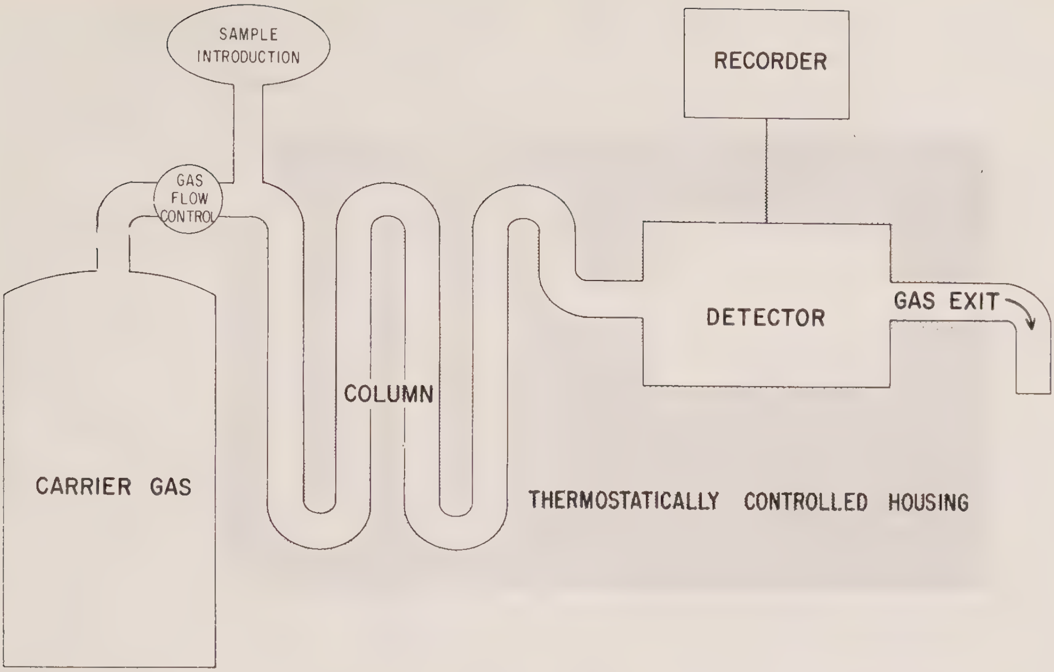

FIGURE I. *DIAGRAM OF GAS CHROMATOGRAPH*

Fig. I

sensitive thermal conductivity detector or the gas density balance, nonspecific changes in the density of the gas or the number and size of the molecules are recorded.

PROCEDURE

Before final separation by gas chromatography, serum fatty acids or other organic acids may be separated into various broad groups by such techniques as thin layer chromatography[2], silicic acid column chromatography[14], [17], centrifugation with acetone precipitation[8], or counter-current distribution[18]. Methyl esters of the fatty acids of the various fractions are obtained by extraction, saponification, and esterification, either with diazomethane[18], or by refluxing with HC1 and methanol[8].

The mixture of methyl esters is then injected into the instrument, and a typical chromatogram with the spike peaks representing specific fatty acid esters can be obtained. Good resolution is evident from sharp peaks with a minimum of tailing. This is due to the choice of optimal system including column, temperature, and detector for the sample and its size. From the chromatogram as automatically recorded, the relative amounts of the various fatty acids in the sample can be calculated as a percentage of the total fatty acids in one of several ways. The amounts are proportional to the peak height, so this measurement may be used. Another method is to calculate the area of the peak by multiplying the width times one-half the height of each peak. Still another is the triangulation method, which is really the width times one-half the height of a triangle drawn to fit the peak's shape. Finally, Lindgren and co-workers[11] have described a computer technique for calculations. The computer method avoids many of the human errors which may occur in the tedious manual methods.

DISCUSSION

Fatty acids may be determined by gas chromatography without esterification, but the liquid phase must be acid to prevent the reactive carboxyl groups from forming soaps. Ester determinations may be carried out at lower temperatures because they are more volatile than the free fatty acids, and in addition, the esters are less likely to be adsorbed by the solid phase. For these reasons, fatty acids are usually determined by GLC as their methyl esters. One can predict the retention time or how fast a fatty acid methyl ester will emerge from the column by plotting a curve with known members of a homologous series. Or, one can qualitatively identify a fatty acid if the retention time is known. The log of the retention time is plotted against the number of carbon atoms and a linear relation is obtained. However, the same linear relationship is also found if the log of the retention time is plotted against the number of double bonds. Therefore, one must beware of a "trap" in an unknown mixture; for example, an 18-carbon fatty acid with two double bonds may emerge before a 20-carbon saturated fatty acid. Separation of saturated and unsaturated fatty acids depends on the type of column, and the retention times also depend on the type of column. With increasing polarity of the coating, analysis time becomes shorter, and peaks become sharper, resolution better.

SOURCES OF ERROR

In Table II are listed various means of avoiding errors in GLC. Two good reviews on practicalities of GLC, from which many of these items have been gleaned, have been presented by Karmen[10] and by Horning *et al.*[6]. Many of these factors must be determined empirically for a given instrument or a particular column, considering the purpose of the experiment. However, some of them apply to any instrument and any experiment. For example, for quantitation the entire sample must be injected into the chromatograph, but this is sometimes difficult to accomplish. The use of an internal standard partially solves this problem. Obviously, if the system is "overloaded," good resolution with well-defined peaks will not be obtained. Overloading is avoided by adjusting the column film to the type of detector (10 per cent liquid or more for thermal conductivity; 5 per cent

TABLE II. HOW TO AVOID ERRORS IN GAS-LIQUID CHROMATOGRAPHIC FRACTIONATIONS OF SERUM FATTY ACIDS

Work within linear range of instrument.
Adjust flow rate proportionally to theoretical number of plates.
Use same recorder chart speed for similar experiments.
Inject entire sample.
Run solvent through GLC to check purity.
Control temperature precisely.
Avoid "bleeding" of liquid phase.
Clean detector frequently to remove pyrolysis products.
Use reference standard each day.
Avoid "overloading."

liquid or less for ionization detectors) and by adjusting the sample size to the type of detector (100-500 μg or more of a 10 to 50 per cent solution for thermal conductivity; 1-50 μg of a 0.1 to 2 per cent solution with at least 0.01 μg of each component for ionization detectors). Temperature and flow rate must be precisely controlled. Temperature and pressure programming, where these factors are changed during the experiment, may be used to obtain better resolution and more effective fractionation.

Serum tends to clog the injection apparatus, and in addition, derivatives of the test substances are often more adaptable to GLC than are the serum constituents per se. Therefore, pre-treatment of the serum is necessary, and it is sometimes difficult to decide which treatment should be used for a particular experiment.

In spite of all the sources of error, it was predicted at a recent symposium[12] that gas liquid chromatography would become one of the most useful instruments in the clinical laboratory in the next decade. At the present time GLC involves a lot of "blood, sweat, and tears." It is not an instrument to plug in, switch on, and sit back until results are recorded.

RESUMÉ OF CLINICAL INTERPRETATIONS

There is a tremendous usage of gas liquid chromatography as a research tool. One of the recent reviewers[1] stated that during the past ten years, GLC has developed and extended at a faster rate than any other analytical procedure in the history of chemistry. Even so, there is only very minimal usage of gas chromatography in the clinical laboratory at the present time, and no methods have thus far proved applicable to routine assay. Currently, many new relationships of organic acids to specific disease states are being investigated. Different investigators have examined the differences in serum fatty acids of healthy subjects and patients with arteriosclerosis, studying the nonesterified fatty acids, the fatty acids of the triglycerides, the fatty acids of the phospholipids, and those of the cholesterol esters. Glaser *et al.*[4] using gas liquid chromatography, identified several branched chain fatty acids in blood. Pietropaolo *et al.*[15] have compared the fatty acid content of lipids in blood with that of exudates and transudates. Serum lipid changes in gynecologic cancer have been identified. Meister and associates[9] have established a method for, and have made extensive studies of, the separation of amino acids by gas liquid chromatography. The determination of Krebs cycle acids by GLC has been reported and provides values comparable to those obtained by the more laborious conventional techniques.

It is most likely that in the future a number of routine uses of gas liquid chromatography will be established in the clinical laboratory. The extremely high sensitivity of the method, the simplicity of the established procedures in other fields, and the applicability of the technique to complex mixtures are all attractive. Identification and measurement of organic acids in serum by gas liquid chromatography is most likely to become an established widely-employed procedure.

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The Measurement of Ammonia in Serum

HARRISON H. LEFFLER, M.D.

Investigation of ammonia levels in biological fluids has become increasingly important in the diagnosis, prognosis, and preventon of ammoniagenic coma. Many methods for the estimation of ammonia have been suggested, the most popular ones being modifications of the microdiffusion process[7, 8]. Difficulties may be encountered with this technique, as it fails to prevent the blood enzymes from liberating ammonia from the serum proteins. Thus, excessively high values may be obtained. Rosenoer[6] states that this artifact constitutes most of the ammonia obtained by diffusion.

Rapid cooling of the sample at the time of collection has been proposed to prevent enzyme action[1, 4, 5]. Conn[1] indicates that the ammonia remains constant for fifteen to forty minutes after collection and that the value in frozen blood remains unchanged for two to three days. Merchant *et al.* found the ammonia to be the same after one hour at room temperature as that in the one-hour quick-frozen specimen. Marshall *et al.*[4] suggest a simple method for quick freezing at -50° C., which allows analysis to be completed, with reliable results, at any time within seventy-two hours. The method to be presented attempts to eliminate these difficulties.

PRINCIPLE

A 12-ml. tube full of blood, collected without stasis, is stoppered and, without waiting for a clot to form, is centrifuged for ten minutes at 3,000 r.p.m. Proteins are precipitated from the clear serum, and the mixture is centrifuged. The ammonia, in an aliquot of the clear centrifugate, is aerated into a tube containing boric acid. Sodium phenate and hypochlorous acid are added to give a stable blue color, the density of which is proportional to the amount of ammonia present. Thus, the ammonia is separated from the proteins before enzyme action has a chance to cause hydrolysis. The final blue color represents true ammonia.

REAGENTS

De-ionized water, free of ammonia, must be used in the preparation of all reagents.

1. *Tungstic Acid Protein Precipitant.* In a 250-ml. volumetric flask containing 100 ml. of water are dissolved 3.5 gm. of sodium tungstate ($Na_2WO_4 \cdot 2H_2O$ – Folin). Seven tenths ml. of concentrated sulfuric acid are added, followed by three drops of phosphoric acid (85 per cent H_3PO_4). The contents are diluted

with water to the 250-ml. mark, mixed and stored at room temperature in a plastic bottle. This reagent is stable indefinitely.

2. *Nitrogen Standard (Stock Solution).* Ammonium sulfate of the highest purity is used, 4.716 gm. being dissolved in water in a one-liter volumetric flask. The contents are diluted with water to the one-liter mark and mixed. 1 ml. = 1 mg. of nitrogen.

Nitrogen Standard (Intermediate). Five ml. of the stock standard solution are placed in a 100-ml. volumetric flask. Water is added to the 100-ml. mark, and the contents are mixed. 1 ml. = 50 μg of nitrogen.

Nitrogen Standard (Working Solution). Four tenths ml. of the intermediate standard are placed in a 100-ml. volumetric flask, and tungstic acid (reagent no. 1) is added to the 100-ml. mark. The contents are mixed and stored in a tightly stoppered plastic bottle. 5 ml. = 1 μg. of nitrogen.

3. *Sodium Hydroxide, 20 Per Cent.* Twenty grams of sodium hydroxide pellets are dissolved in water to a volume of 100 ml. The solution is stored in a plastic bottle at room temperature.

4. *Sodium Hydroxide, 2.5 Per Cent.* Two and one half grams of sodium hydroxide pellets are dissolved in water to a volume of 100 ml. The solution is stored in a plastic bottle at room temperature.

5. *Boric acid, 0.25 Per Cent.* Two-and-one-half grams of boric acid crystals (H_3BO_3) are dissolved in water to a volume of 1000 ml.

6. *Phenol solution, 2.5 Per Cent.* Two and one half ml. of melted phenol are transferred by a warmed pipet to 80 ml. of water in a 100-ml. volumetric flask. As soon as solution is complete, water is added to the 100-ml. mark and mixed. Stored at room temperature in a plastic bottle away from light, it is stable for several months.

7. *Sodium Nitroprusside.* One hundred mg. of sodium nitroprusside are dissolved in 10 ml. of water and preserved in a small amber-colored bottle. This is stable for several weeks at room temperature.

8. *Sodium Hypochlorite.* The chlorine content of this is 4 to 6 per cent, and it is stored in a plastic bottle in a refrigerator.

SPECIAL APPARATUS

1. *Wash bottle:* A four-ounce bottle with a mouth sufficiently wide to accommodate a six-hole rubber stopper.

2. *Aeration bottles* (quantity sufficient for the amount of work being done): Glass tubes measuring about 10 cm. in length with the inside diameter (about 25 mm.) large enough to accommodate a No. 6 two-hole rubber stopper.

3. *Matched cuvets:* Glass tubes measuing 125 x 15 mm.

4. *Glass head with six-hole rubber stopper* connected to a vacuum pump.*

5. *Rubber stoppers:*
   (a) Six-hole rubber stoppers for the wash bottle and glass head.
   (b) Two-hole rubber stoppers for the aeration bottles and matched cuvets (No. 0 and No. 6).

6. *Rigid plastic tubing* to fit the various rubber stoppers, measuring approximately 145 mm. in length with the inside diameter measuring about 2 mm. (the air inlet tubes from discarded transfusion sets are satisfactory). A quantity of one-inch lengths from this same tubing is also used.

7. *Flexible plastic tubing* to fit the rigid tubing (used to connect the various elements of the aeration apparatus.)

ASSEMBLY OF AERATION APPARATUS

Into the wash bottle are placed 60 ml. of approximately 1N sulfuric acid. The six-hole rubber stopper containing the the long inlet tube is inserted in the bottle with the tube projecting well below the surface of the acid. One-inch rigid plastic tubes are inserted in the stopper holes that are to be used for aeration, glass plugs being inserted in the remaining holes.

In an aeration bottle is placed a two-hole rubber stopper containing a plastic inlet tube which extends to within 5 mm. of the bottom of the tube. A one-inch plastic tube is placed in the remaining hole. Each cuvet is similarly assembled with the air inlet tube reaching to within 5 mm. of the bottom of the tube. The first unit of the aeration bottle-cuvet set is labelled "B" (for blank), this label being placed on both the aeration bottle and the corresponding cuvet. In a similar manner, the second unit is labelled "S" (for standard), and the units containing the unknowns are labelled in the appropriate sequence.

The exit tube from the wash bottle is connected by the flexible plastic tubing to the inlet tube of the aeration bottle; the exit tube from the aeration bottle is then connected to the inlet tube of the cuvet, the cuvet finally being connected to the glass head of the vacuum pump. Each unit is connected in this manner.

METHOD

Venous blood (10.0 ml.) is collected without stasis and placed in a 12-ml. tube which is stoppered. Without waiting for clotting, the blood is centrifuged at 3000 r.p.m. for five minutes. Two ml. of the clear serum are pipetted into a 12-ml. tube, and 8.0 ml. of tungstic acid solution are added. The tube is stoppered and inverted several times to mix the contents. Centrifuging for five minutes at 3000 r.p.m. should give a water-clear supernatant. Five ml. of the clear supernatant are pipetted into an aeration bottle labelled "U." Into a second aeration bottle labelled "S" are placed 5.0 ml. of the working standard solution, and into a third labelled "B" (for blank), are placed 5.0 ml. of tungstic acid protein precipitant. Into each of three matched cuvets labelled "B," "S" and "U" are pipetted 2.0 ml. of boric acid solution. The tubes and bottles are tightly stoppered with each inlet tube extending to about 5 mm. from the glass bottom.

The exit tube of the aeration bottle labelled "B" is connected to the inlet tube of the cuvet labelled "B," and the exit tube of the cuvet is connected to the vacuum pump. With a bulb pipet, 1.0 ml. of 20 per cent sodium hydroxide is added through the inlet tube to the aeration bottle, and the inlet tube is quickly connected to the exit tube of the wash bottle. The standard and unknown units are assembled and treated in a similar manner. Glass plugs are inserted in all unused holes in the six-hole rubber stoppers. The vacuum pump is started, and aeration is continued for fifteen minutes. During the fifteen-minute aeration period, fresh phenol nitroprusside and sodium hypochlorite solutions are made as follows:

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*Blood pipet cleaning outfit. Scientific Products Co., No. B-4080

In a 15-ml. test tube are placed 10.0 ml. of 2.5 per cent phenol, followed by 0.1 ml. of the 1-per cent sodium nitroprusside solution. The tube is inverted to mix and is then ready for use.

In a second 15-ml. test tube are placed 10.0 ml. of 2.5 per cent sodium hydroxide. Two tenths ml. of sodium hypochlorite is added and mixed. The solution is then ready for use.

At the end of fifteen minutes, the vacuum pump is stopped, and the cuvets are disconnected. The stopper in the cuvet labelled "B" is loosened but not removed. It is raised slightly, and 1.0 ml. of the fresh phenol nitroprusside is added, quickly followed by 1.0 ml. of the sodium hypochlorite. With the stopper and inlet tube replaced, the contents of the cuvet are rapidly and thoroughly mixed. The stopper is removed and replaced by a solid rubber stopper; the cuvet is set aside. Each cuvet, in turn, is treated in a similar manner. After standing fifteen minutes at room temperature, maximum color has developed.

With the colorimeter set at a wave length of 625 $m\mu$ and the blank set to read 100 per cent transmittance, the standard and unknowns are read, and the results are recorded.

CALCULATION

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 1 = \mu\text{g. ammonia nitrogen per ml. of serum.}$$

DISCUSSION

Deionized water, 2 ml., when treated with 1.0 ml. of fresh phenol nitroprusside, solution followed by 1 ml. of fresh sodium hypochlorite, should give no color. Freshly prepared tungstic acid protein precipitant treated similarly and read in the colorimeter against a water blank should give a reading very close to 100 per cent transmittance. Repeated exposure of reagents to the air of the general laboratory may cause contamination with ammonia and result in the reagent blank reading below 90 per cent transmittance. Should this occur, a new reagent must be prepared.

Phenol nitroprusside and sodium hypochlorite solutions, when made fresh just before use, give a much greater color density than the same solutions used after storage in the refrigerator. These reagents, as suggested by Chaney and Marbach[3] have therefore been modified.

During color development, the tubes should be protected from direct daylight. The usual artificial light of the laboratory seems to have no effect[3].

The fifteen minutes of aeration, with the apparatus used, has been sufficient. However, each laboratory should determine the time needed to give the greatest color density.

SOURCES OF ERROR

Contamination of glassware or reagents by ammonia in the air is the greatest source of error. A room in which urinalyses are being made should never be used for blood ammonia determinations. Interfering amounts of ammonia in the laboratory air may be detected as follows: Non-filtered air is passed through 2 ml. of boric acid reagent in a matched cuvet for the usual time of the aeration period. Phenol nitroprusside and sodium hypochlorite reagents are added and the tube compared with 2 ml. of the same, nonaerated, boric acid in a second cuvet to which the same color reagents have been added.

All glassware should be thoroughly washed and dried in the hot air oven and used as soon as possible after cooling.

With careful technic, the standard deviation should be not over ± 0.04 μg per ml. of serum.

Should the serum sample give a density too great to be read, appropriate dilutions should be made.

NORMAL VALUES

Normal values vary markedly among different workers, the reason being largely due to the many different methods being used. Some workers give their normals as varying between 0.2 μg. and 0.8 μg. per milliliter. Others obtain a range between 1.0 μg. and 3.0 μg. per milliliter. Using the technic presented, the range has been between 0.2 μg. and 1.0 μg. per milliliter. It is therefore necessary for each laboratory to establish its own range of normal values.

Other factors should be considered in accounting for the great variation of normal values. Blood ammonia is derived from three sources: (a) enzyme action on proteins and urea in the intestinal tract; (b) secretion of NH_3 by the kidneys; and (c) changes produced by muscle activity. This third source is one very important cause of variations. Blood taken after the muscles of the arm have been contracted by doubling the fist give values which are about double those taken without stasis and with the arm relaxed.

In patients suspected of having liver disease but who show normal blood ammonia values, a tolerance test has been suggested[2]. Conn gives three grams of ammonium chloride followed by 250 ml. of water. Forty-five minutes later, blood is drawn for analysis. He finds no elevation in normal individuals, in obstructive jaundice, fatty infiltration, and in most cases of acute hepatitis. In 83 per cent of sixty patients with cirrhosis, blood ammonia values were elevated, being related to the degree of portal collateral circulation.

SUMMARY

The collection of blood under ideal conditions, without stasis and with careful attention to the details of the technic of the method presented, should give reliable results.

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The Measurement of Carbon Dioxide and pCO_2 in Serum

KURT M. DUBOWSKI, Ph.D.

A. Gasometric Method for Carbon Dioxide in Serum

INTRODUCTION

Determination of the carbon dioxide content of serum is one of the most commonly performed procedures in clinical chemistry because of its fundamental importance in the assessment of the acid-base status. While many analytical procedures have been developed for this analysis, *gasometric* methods possess the following significant advantages for clinical applications: (a) reliability (i.e., accuracy, precision, and specificity); (b) rapidity; (c) simplicity of apparatus and technique; (d) minimal specimen requirements.

The classical technics for gasometric analysis of blood gases were largely developed by Van Slyke[1] and his associates and involve isolation of a measured sample, addition of acid, vacuum extraction of the liberated gases, and measurement of the gases volumetrically or manometrically, usually after selective gas absorption by added reagents. The method presented employs the Natelson microgasometer[2], which combines most of the desirable features of the Van Slyke and Neill manometric apparatus[3] with the capability to analyze ultra-micro specimens.

PRINCIPLE

The carbon dioxide of a measured volume of serum is released by addition of lactic acid, and extracted with agitation under reduced pressure. The resulting gas pressure (P_1) is measured manometrically at a fixed volume. The CO_2 is absorbed with sodium hydroxide, and the resulting gas pressure (P_2) is again measured at the same volume. The carbon dioxide content of the specimen is calculated from the difference in P_1 and P_2 and factors appropriate to the temperature and the sample volume.

REAGENTS

1. *Carbon Dioxide Standard, 25 mM Per L.* Anhydrous reagent grade sodium carbonate is dried for three hours at 200° C and allowed to cool to room

temperature in a desiccator. Two hundred sixty-five mg. are transferred quantitatively to a 100-ml. volumetric flask, diluted to the mark with CO_2-free water, and mixed. The standard should be stored over mercury or in sealed ampules to prevent absorption of atmospheric CO_2.

2. *Carbon Dioxide-free Water.* Freshly distilled water is passed through a suitable mixed-bed ion exchange column, or may be degassed by boiling for five minutes and allowing it to cool in a closed container.

3. *Lactic Acid, Approximately 1 Normal.* Ninety ml. of reagent grade 85 per cent lactic acid are diluted to 1 l. with CO_2-free water.

4. *Octyl Alcohol, Reagent Grade.*

5. *Sodium Hydroxide, Approximately 3 Normal.* One hundred and twenty grams of reagent grade sodium hydroxide are dissolved in CO_2-free water and diluted to 1 l.

Note: Small volumes of these reagents are dispensed for daily use in screw-capped vials which contain approximately 5 ml. of mercury.

SPECIAL APPARATUS

Natelson Microgasometer, Model 600.* The basic Model 600 microgasometer can be equipped with an accessory motorized shaker attachment (Cat. No. M-373-20), or may be replaced by a motorized microgasometer (Model 650).

PROCEDURE

Collection and Preparation of Specimens

1. Blood serum from capillary, venous, or arterial blood can be used. Venous blood should be drawn with minimum stasis and after the tourniquet has been released. Capillary blood specimens must be collected without air bubbles from deep skin punctures and without excessive squeezing of tissues. It is convenient to collect about 0.2-ml. specimens of capillary blood directly into 75-mm. long capillary tubes of 2.0 mm. I.D., which are then closed at both ends with clay or plastic caps.

2. Venous blood specimens should be placed into small glass tubes and stoppered immediately, and the serum separated from the clot without delay. It can then be preserved in closed micro test tubes for several hours at room temperature without change in CO_2 content.

3. All blood specimens should be centrifuged briefly in closed containers, since up to 8 per cent of the water content and most of the dissolved CO_2 can be lost through the forced draft.

Analysis of Carbon Dioxide

1. Approximately 0.1 ml. or less serum is placed into a 6 x 50 mm. micro test tube containing 0.5 ml. of mercury, and the tube is stoppered.

2. The microgasometer is checked for absence of leaks, water, and air bubbles. The reagents are checked for presence of adequate mercury in each vial.

3. The reagent blank (usually 0 to 2 mm. Hg) is determined by following steps 4 to 9, substituting 30 μl. of lactic acid for the serum specimen.

4. The following are drawn into the microgasometer reaction chamber in the order given; the tip is wiped with cellulose tissue or gauze between additions; and the stopcock is then closed:

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*Scientific Industries, 220-05 97th Ave., Queens Village, New York.

(a) 30 µl. serum followed by a 10 to 20 µl. mercury plug.

(b) 30 µl. 1 N lactic acid, followed by a 10 to 20 µl. mercury plug.

(c) 10 µl. octyl alcohol, followed by a 10 to 20 µl. mercury plug.

(d) 100 µl. CO_2-free water followed by mercury to the 0.12 ml. mark on the reaction chamber.

5. The chamber is evacuated until the mercury meniscus reaches the lower one-third of the chamber. The chamber is shaken mechanically or intermittently by hand for one minute, and the mercury meniscus then lowered to the 3-ml. mark on the reaction chamber.

6. The liquid (not mercury) meniscus is immediately adjusted to the 0.12-ml. mark. The manometer reading, P_1, is recorded in millimeters, as is the temperature.

7. The vacuum is eliminated by raising the mercury column to the top of the manometer; the reaction chamber stopcock is opened; and 30 µl. of 3 N sodium hydroxide are drawn into the reaction chamber, followed by mercury to the 0.12-ml. mark. The stopcock is closed.

8. The mercury meniscus is lowered to the 3.0-ml. mark and immediately brought to the 0.12-ml. mark. The manometer reading, P_2, is recorded in millimeters.

9. The instrument is cleaned by eliminating the vacuum then expelling all fluid, rinsing successively with about 0.15 ml. of water, 0.15 ml. of 1 N lactic acid, and 0.15 ml. of water, and eliminating all traces of water. The gasometer is secured with the reaction chamber stopcock open and mercury filling the instrument to the 100 µl. pipette mark.

CALCULATIONS

1. Serum Carbon Dioxide mM/L. = $(P_1 - P_2 - \text{Reagent Blank}) \times F_t$

where F_t is the factor appropriate to the test temperature from Table I.

EXAMPLE: Reagent Blank = 2 mm.
P_1 = 178 mm.
P_2 = 80 mm.
Temperature = 25° C
Serum Carbon Dioxide =
$(178 - 80 - 2) \times 0.232 = 22.3$ mM/L.

TABLE I CARBON DIOXIDE FACTORS

TEMPERATURE ° C.	CO_2 FACTOR, F_t IN mM PER LITER
18	0.240
19	0.238
20	0.237
21	0.236
22	0.235
23	0.234
24	0.233
25	0.232
26	0.231
27	0.230
28	0.229
29	0.228
30	0.227
31	0.225
32	0.224
33	0.222
34	0.221
35	0.219

DISCUSSION

The basic procedure determines the concentration of total CO_2, which includes the dissolved CO_2 as well as that present as H_2CO_3 and HCO_3^-. It is now recognized that, with the methods presently available for drawing and analyzing blood, it is simpler and more accurate to determine the CO_2 content of serum or plasma than to perform determinations of CO_2 capacity[4].

The average time required for a single analysis is six to eight minutes, including clean-up. The labor required, though not the elapsed time, can be reduced by using a motorized shaker attachment with timer, which eliminates the need for manual shaking of the microgasometer during the gas evolution phase. Where many analyses of serum carbon dioxide are performed, it is convenient to use a fully motorized microgasometer. Such an instrument was described by O'Mara and Faulkner[5] and is available in commercial form. Improvements in the original Natelson[2] procedure for microgasometry have been described by Natelson and Menning[6] and by Holaday and Verosky[7].

The method described has good precision, with a standard deviation of ± 0.24 mM per l. in routine use[7, 8]. The accuracy of microgasometric analyses should be checked periodically by analyzing the 25 mM. per l. carbon dioxide standard. In such standard analyses, the octyl alcohol is replaced by lactic acid, because of the variable solubility of CO_2 in the alcohol[9].

SOURCES OF ERROR

Drawing and processing of the blood specimen for the measurement of CO_2 may lead to greater potential errors than the analysis. Samples should not be collected, stored, or centrifuged under oil since CO_2 is quite soluble in oil[10]. Presence of volatile substances in the serum (e.g., anesthetic gases) can yield falsely elevated results, while failure to separate the serum promptly from the cells can cause a pH shift with a consequent drop of about 5 per cent in the total CO_2[11]. The most common source of errors in the apparatus is air leaks. The reagents become easily contaminated in daily use and should be renewed frequently when kept in small vials over mercury for convenience.

RANGE OF NORMAL VALUES

The concentration of CO_2 of venous serum is 24 to 34 mM. per l. for normal adults[11, 12].

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B. Graphical Computation of Carbon Dioxide Tension in Serum

INTRODUCTION

Estimation of serum pCO_2 ($=$ partial pressure of CO_2 $=$ carbon dioxide tension) by solution of the Henderson-Hasselbalch equation or by interpolation methods can be greatly simplified by use of appropriate nomograms or other charts. Use of graphical computation techniques is generally employed for routine clinical application of pCO_2 estimation with the Henderson-Hasselbalch equation, unless pre-calculated tables are used for the computation. With interpolation methods, graphical computations avoid logarithms and thus expedite and simplify the calculation.

INTERPOLATION METHODS

CO_2 content versus pCO_2. Estimation of the serum or plasma pCO_2 can be performed by interpolation in a plot of pCO_2 against either total CO_2 or pH. In 1923, Peters, Eisenman, and Bulger[1] and Peters[2] reported that a plot of the logarithm of CO_2 tension against the logarithm of total CO_2 of whole blood, separated serum, or "true" plasma approximates a straight line. On the basis of this relationship, Eisenman[3] developed a method for estimating the pCO_2 from CO_2 analyses.

The actual CO_2 absorption "curve" of the serum is located on the logarithmic CO_2 absorption chart from the results of three analyses of serum or plasma total CO_2. One analysis is performed at once on the anaerobically separated sample, and two others after equilibration of the sample at 38° C. with gas mixtures of known CO_2 tensions below and above that of circulating blood. (Ordinarily, CO_2 tensions of about 30 and 60 mm. Hg. are used for equilibration.)

Typical results are shown plotted as points A and B on Peters' logarithmic chart[4] (Figure 1). Point X denotes the total CO_2 content of the original serum sample as separated from the cells, and the abscissa of this point indicates the serum CO_2 tension, 41 mm. Hg.

pH versus pCO_2. Methods were developed independently by Astrup[5], Brewin *et al.*[6], and Peirce[7] for the derivation of plasma or serum pCO_2 by interpolation in a plot of log pCO_2 against pH, based on the principle that the relationship between log pCO_2 and pH of whole blood, true plasma or separated serum within the pH range of 7.2 to 7.6 is approximately a straight line, which may be determined from two known points. The actual pH of a whole blood, plasma or serum sample is measured. The sample is then equilibrated at 38° C. with a pair of gas mixtures of known pCO_2 which, ideally, will bracket the unknown pCO_2 of the sample. The pH of the sample is measured after each equilibration, and the buffer line of the specimen drawn on a semi-logarithmic plot. The original sample pH is interpolated on this line and the original sample pCO_2 derived from this point. On the typical pH/pCO_2 plot[8] (Figure 2), points A and B represent the measured pH after equilibration of the blood sample at pCO_2 $=$ 60 mm. Hg and pCO_2 $=$ 25 mm. Hg,

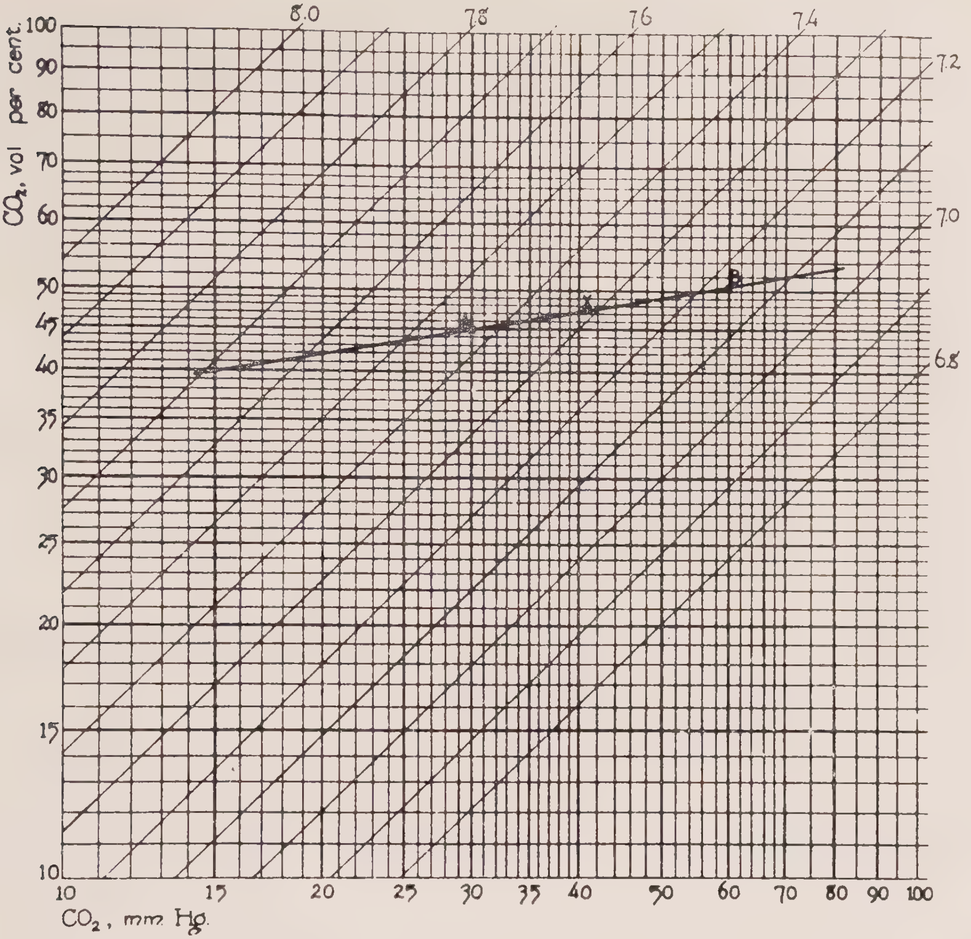


Fig. 1. Logarithmic Carbon Dioxide Absorption Plot for Eisenman's Interpolation Method for pCO_2. (Reproduced from Peters and Van Slyke[4] by courtesy of The Williams & Wilkins Co.)

respectively. The original specimen pH of 7.15 is shown as Point C on the buffer line and its ordinate indicates the original sample pCO_2 of 52 mm. Hg. The characteristic slope of the buffer line is a function of the buffer capacity of the specimen, and is therefore related to hemoglobin concentration in a whole blood sample and protein concentration in plasma or serum samples.

Under favorable circumstances the coefficient of variation for estimation of pCO_2 by this method is ±2 to 2.5 per cent[9–11]. If the actual pCO_2 of whole blood is desired, the measurements should be carried out on whole blood or on "true" plasma separated anaerobically and promptly at 38° C.[5]

GRAPHICAL AND MECHANICAL SOLUTION OF THE HENDERSON-HASSELBALCH EQUATION

The Henderson-Hasselbalch[12] equation, a special instance of the Law of Mass Action for a single phase system such as whole blood or plasma, demon-

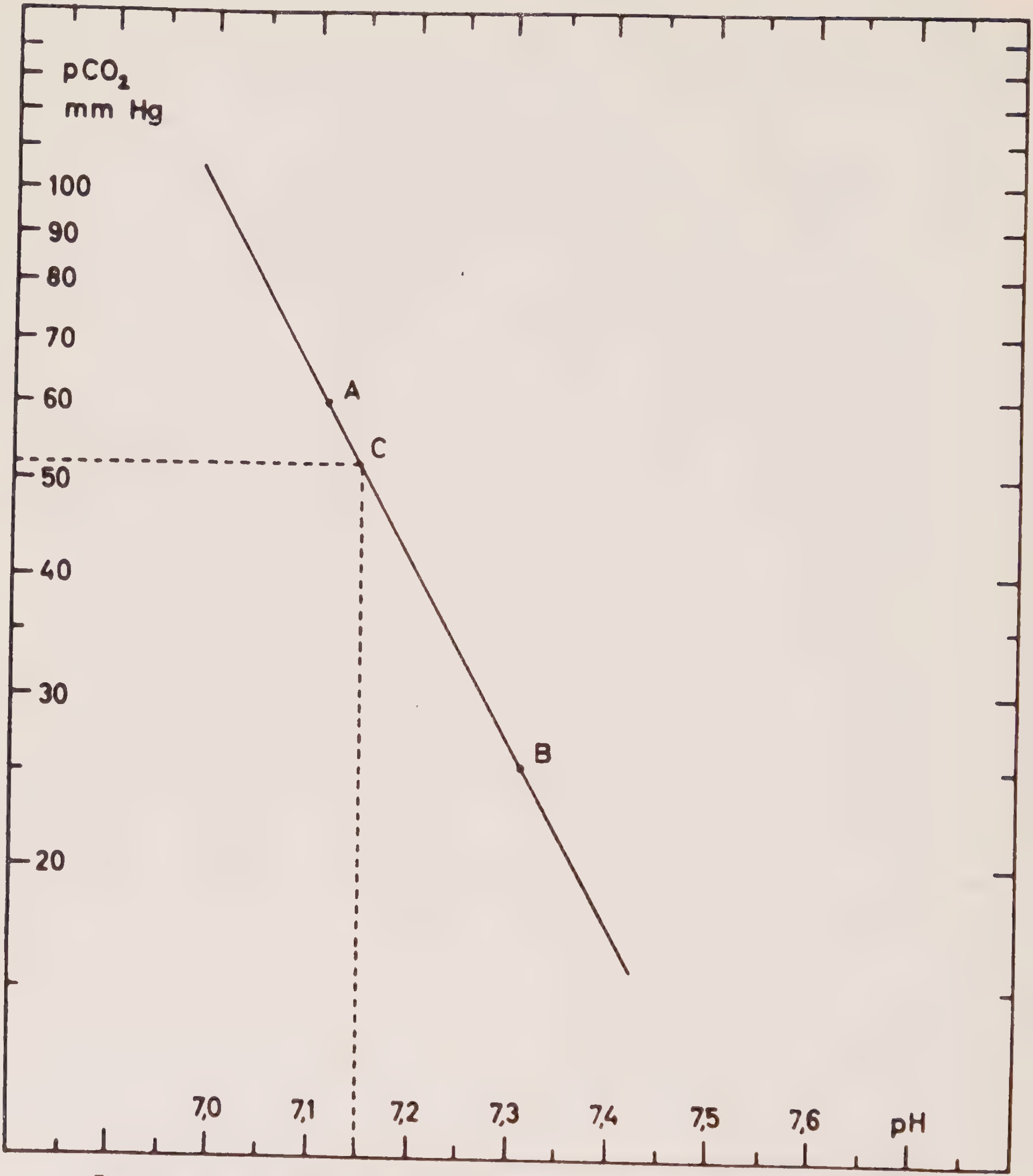


Fig. 2. pH/log pCO_2 Plot for Astrup's Interpolation Method for pCO_2. (Reproduced from Astrup and Siggaard-Andersen` by courtesy of the authors and Academic Press, Inc.)

strates that blood or plasma pH is governed by the ratio of combined CO_2 to free CO_2. It is now usually represented as

$$pH = pK + \log \frac{(HCO_3^-)}{(H_2CO_3)} \qquad I$$

where (H_2CO_3) represents the total concentration of free carbonic acid, whether in the form H_2CO_3 or dissolved CO_2[13]. The free carbonic acid concentration is not readily measured but is in equilibrium with, and proportional to, dissolved CO_2, which is, in turn, directly proportional to the partial pressure of CO_2 (pCO_2). Therefore, a more practical form of the equation is

$$pH = pK' + \log \frac{(HCO_3^-)}{\alpha \bullet pCO_2} \qquad \text{II}$$

where pK' = first dissociation constant of H_2CO_3
α = solubility coefficient of CO_2.

Since further

$$\text{Total } CO_2 = (HCO_3^-) + (H_2CO_3 \text{ including dissolved } CO_2) \qquad \text{III}$$
$$\text{and } (HCO_3^-) = \text{Total } CO_2 - \alpha \bullet pCO_2, \qquad \text{IV}$$

Equation II can be rewritten

$$pH = pK' + \log \left[\frac{\text{Total } CO_2 - \alpha \bullet pCO_2}{\alpha \bullet pCO_2}\right] \qquad \text{V}$$

In this form, it can serve for calculation of the pCO_2 where the pK' and α values are known for a given set of sample conditions and total CO_2 is experimentally determined on the original sample. Substituting generally accepted values [14–19, 25] in equation V yields

$$pH = 6.10 + \log \left[\frac{\text{Total } CO_2 - 0.03\ pCO_2}{0.03\ pCO_2}\right] \qquad \text{VI}$$

when total CO_2 is expressed in mMoles per l. and pCO_2 in mm. Hg. Solving for pCO_2 yields

$$pCO_2, \text{ mm. Hg} = \frac{\text{Total } CO_2, \text{ mM per L.}}{0.03\ [\text{antilog}\ (pH - 6.10) + 1]} \qquad \text{VII}$$

HENDERSON-HASSELBALCH FACTOR TABLES

Use of equations VI and VII for calculation of pCO_2 is usually considered too laborious for routine laboratory applications and consequently various labor-saving schemes have been devised to simplify and expedite this task. Tables of factors have been published, which reduce the necessary operations to 1) determination of the anaerobically separated plasma or serum total CO_2 content, 2) determination of the whole blood or "true" plasma pH at 38° C., and 3) solving an equation of the form

$$pCO_2,\ \text{mm. Hg} = \frac{\text{Total } CO_2,\ \text{mM/L}}{F} \qquad \text{VIII}$$

where F is a factor consisting of the denominator of Equation VII, corresponding to the measured pH, and is obtained from the table of Møller[20] or Leitner and Thaler[21] or a similar compilation.

NOMOGRAMS

Among early approaches to simplifying the solution of the Henderson-Hasselbalch equation for calculation of the pCO_2 was the use of nomograms or alignment charts. In 1928, Van Slyke and Sendroy[19] published a d'Ocagne or alignment nomogram with scales representing total CO_2 (in both mM./L. and volume per cent), Bicarbonate (in mM./L.), pH, H_2CO_3 (in mM/L.), and CO_2 tension (in mm. Hg). A straight line intersecting any two scales at experimentally determined values intersects the other scales at points which correspond to simultaneously occurring values and thus permits direct reading of the pCO_2 from total CO_2 and pH determinations. The authors suggested determining CO_2 content on plasma or serum with precautions to prevent loss of CO_2 in the separation and determining the pH on whole blood, thus assum-

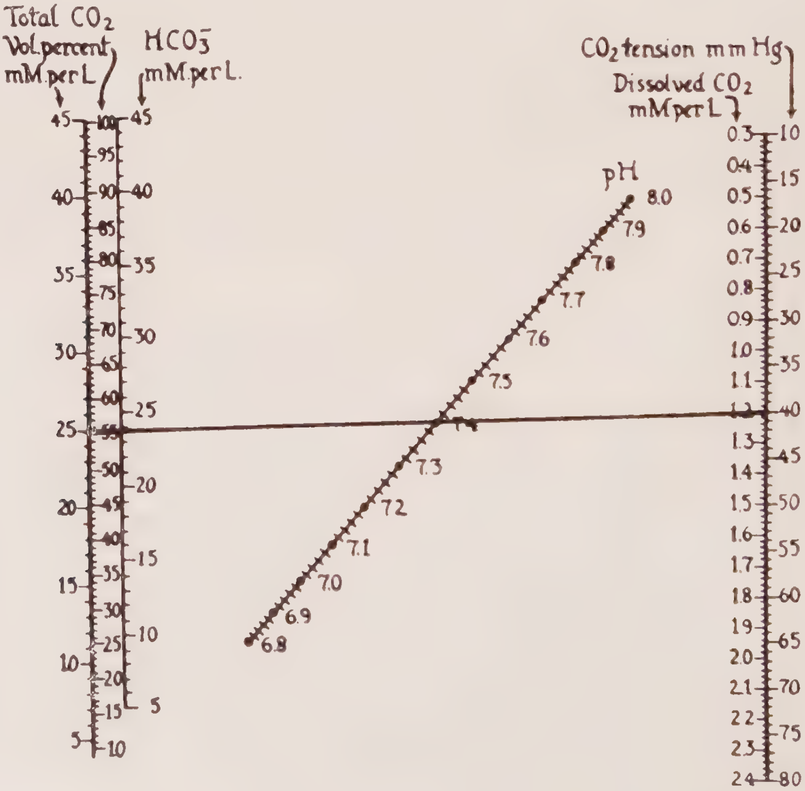


Fig. 3. McLean's Nomogram for Solution of the Henderson-Hasselbalch Equation. (Reproduced from McLean[23] by courtesy of the American Physiological Society)

ing normal distribution of CO_2 between plasma and erythrocytes, and pointed out that the chart was only approximate, but closely so, for calculations involving the concentration of total CO_2.

In a detailed discussion of laboratory and clinical concepts in acid-base balance, Singer and Hastings[22] included a 7-scale nomogram for human blood acid-base factors. It contains plasma CO_2, plasma bicarbonate, plasma pH and plasma pCO_2 scales, and like the Van Slyke and Sendroy nomogram, can be used to estimate pCO_2 directly from the results of analyses for any two of the other factors.

McLean[23] presented a revised version of the Van Slyke and Sendroy nomogram (Figure 3), which has found wide application for graphical computation of the pCO_2 from the results of total CO_2 and pH determinations. The straight line in Figure 3, as an illustration, intersects the average normal values of blood pH (7.40) and plasma or serum total CO_2 (25 mM. per L.). The corresponding CO_2 tension is indicated as approximately 40 mm. Hg, compared to a value of 39.8 mm. Hg, obtained by solving equation VII, inserting the same pH and total CO_2 values.

Siggaard-Andersen[24] more recently published an acid-base nomogram very similar to the Van Slyke and Sendroy chart (Figure 4). The chart consists of a linearly graduated straight line scale for blood (or plasma) pH and a parallel straight line pCO_2 scale logarithmically graduated in the opposite direction, and was calculated from the Henderson-Hasselbalch equation (Equation II), employing a plasma CO_2 solubility coefficient (α) of 0.0300 mM. per/L. per mm. Hg. at 38° C. and a first dissociation factor for H_2CO_3 (K') of 6.099 at a pH of 7.40 and at 38° C. As illustrated by the line superimposed on Figure 4, with a blood (or plasma) pH of 7.40 at 38° C. and a plasma total CO_2 of 25 mM. per L., the chart yields a pCO_2 of 40 mm. Hg. The nomogram was computed for values at 38° C., but can be employed without significant differences with values obtained at 37° C. If the patient's temperature deviates more than 2° C. from 38° C., the pCO_2 value should be corrected to the patient's temperature by the formula[24, 25]:

$$pCO_2^{T} = \text{antilog}\left[\log pCO_2^{38^\circ} - 0.021\,(38^\circ - T)\right] \qquad \text{IX}$$

$$\text{where } T = \text{Patient's temperature, } ^\circ C$$

SLIDE RULES

A special-purpose slide rule for solving the Henderson-Hasselbalch equation has been proposed by Searcy, Bergquist, and Tomeoni[26] who developed it for the following form of that equation:

$$pH = pK' + \log \frac{(\text{Serum } HCO_3^-)}{(\text{Dissolved } CO_2)} \qquad \text{X}$$

The same principle could be applied to Equation VI or VII by substituting total plasma or serum CO_2 and whole blood pH^{38° scales for the serum bicarbonate and dissolved CO_2 scales of the slide rule of Searcy and associates.

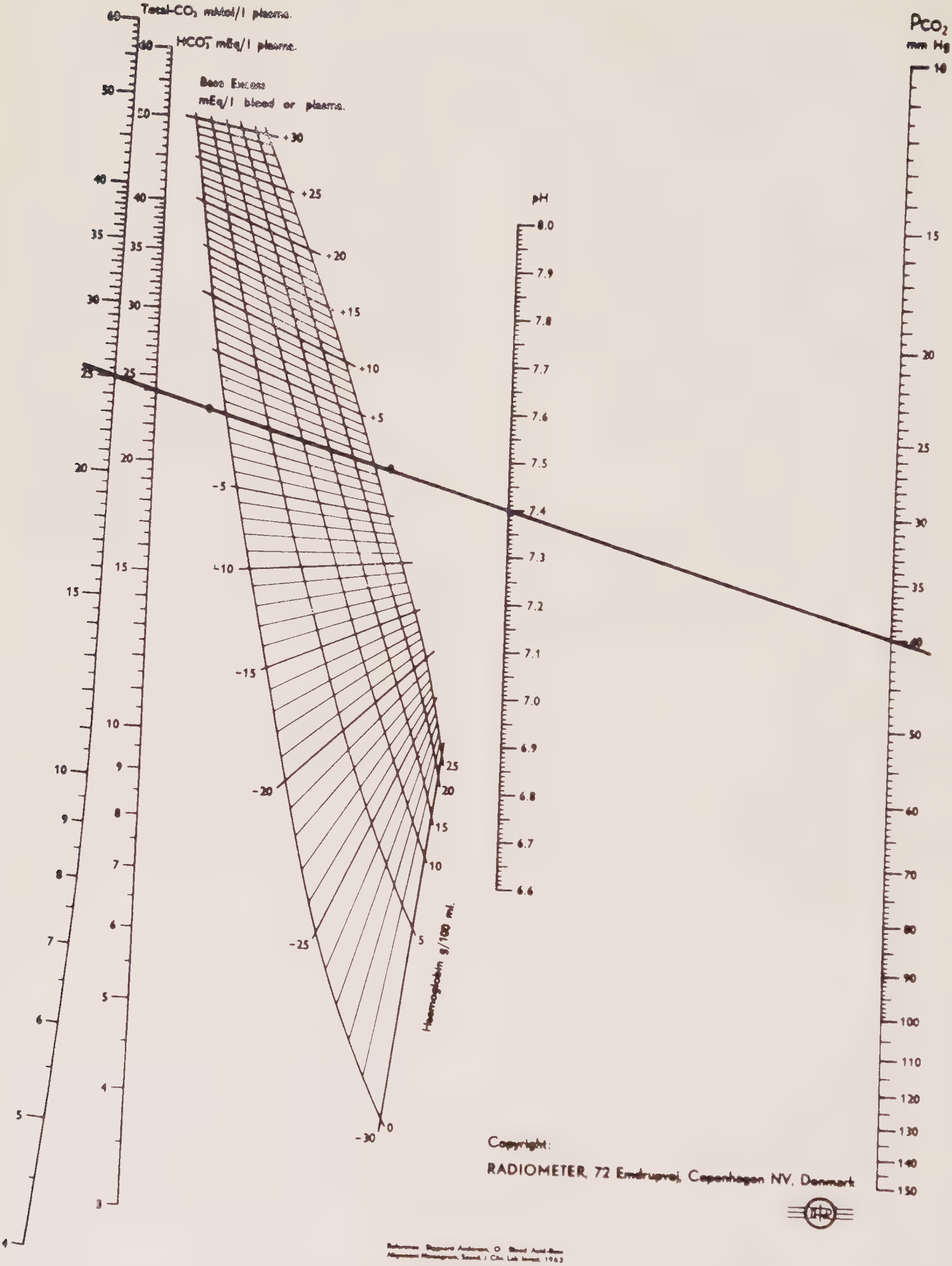


Fig. 4. Siggaard-Andersen's Nomogram for Solution of the Henderson-Hasselbalch Equation. (Reproduced from Siggaard-Andersen[24] by courtesy of Radiometer)

RÉSUMÉ OF CLINICAL APPLICATIONS

The Henderson-Hasselbalch equation was originally used for calculation, from alveolar pCO_2 and blood total CO_2, of the blood pH since direct measurement of pH was technically difficult at the time. A later application was the calculation of the pCO_2 from the measured pH and the gasometrically determined total CO_2 value. The principal purpose of the graphical methods for computation of pCO_2 from measurements of blood, plasma or serum pH and CO_2 is also the estimation of the existing sample pCO_2 when direct methods for its determination (e.g., by electrometric measurement) are not available. The role of such carbon dioxide tension (pCO_2) estimations in the evaluation of the clinical acid-base status is, however, complex and many-sided.

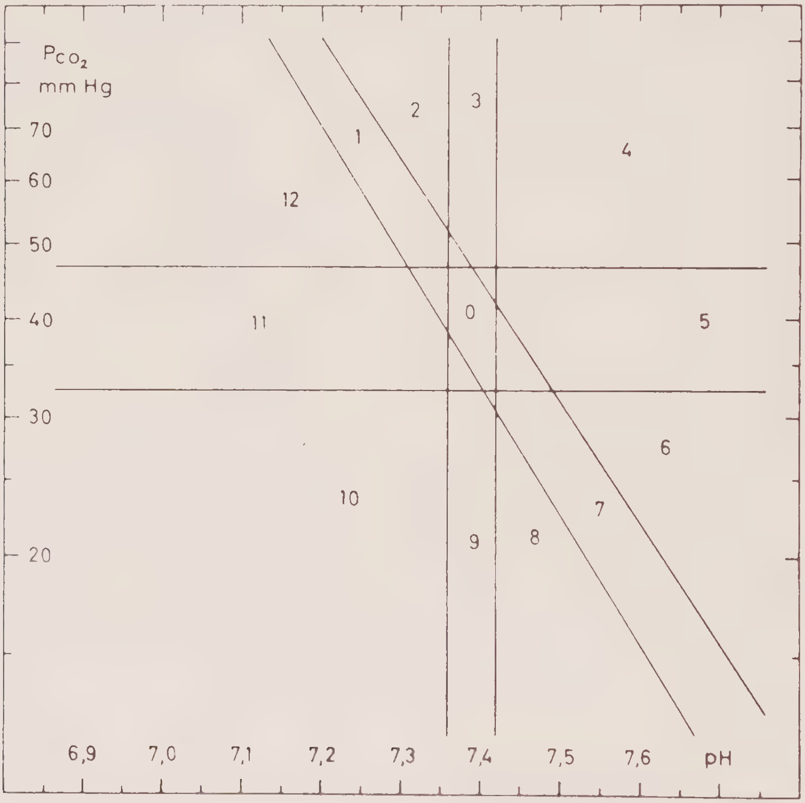
The intracellular, and to a slightly lesser extent the extracellular, integrity of the human body must be maintained with relative constancy with respect to such physical conditions as osmolality, temperature and pH, if the vital metabolic processes are to continue. The principal buffer systems of the blood are hemoglobin and the bicarbonate-carbonic acid system. The latter also buffers interstitial and tissue fluids. The relationship between the pH of blood and the bicarbonate-carbonic acid buffer system is described by the Henderson-Hasselbalch equation (Equation I). To express the acid-base status in terms of the bicarbonate-carbonic acid buffer system according to the Henderson-Hasselbalch equation, three acid-base values of blood are relevant: (a) the pH; (b) the pCO_2, and (c) a value for the nonrespiratory component, such as HCO_3^- concentration or total CO_2 concentration. The pCO_2 is a measure of alveolar ventilation and, therefore, of the respiratory disturbances of the acid-base metabolism. It is, in fact, the most convenient index of alveolar ventilatory effectiveness.

Four major classifications are generally employed in describing disturbances of the acid-base relationship: respiratory acidosis, metabolic acidosis, respiratory alkalosis, and metabolic alkalosis. Table I reflects the pCO_2 deviations which are commonly associated with each of these four disturbances. It is evident that measurement of the pCO_2 alone is inadequate for differentiation of these forms, particularly since "pure" respiratory or metabolic disturbances are usually altered by compensatory body responses. Less

TABLE I CARBON DIOXIDE TENSION IN MAJOR ACID-BASE DISTURBANCES

DISTURBANCE	PRIMARY DEFECT	pCO_2 FINDING
Respiratory Acidosis	Retention of CO_2	Increased pCO_2
Respiratory Alkalosis	Excess CO_2 loss from hyperventilation	Decreased pCO_2
Metabolic Acidosis	Retention of fixed (non-volatile) acids	Decreased pCO_2
Metabolic Alkalosis	Loss of hydrogen ions	Increased pCO_2

commonly, there also occur simultaneous primary respiratory and non-respiratory (metabolic) disturbances. The acid-base disturbances can generally be recognized and classified into one of the four classical categories from simultaneous estimation of two of the three major values (pH, pCO$_2$, bicarbonate or total CO$_2$ concentration) and measurement or computation of the third. The terms *hypercapnia* and *hypocapnia* are occasionally employed to designate, respectively, abnormally increased and abnormally reduced carbon dioxide tension in the arterial blood and hence in the tissues.



Each area in the diagram represents a specific state of acid-base disturbance:

0 = Normal region.
1 = Uncompensated respiratory acidosis.
2 = Partly compensated respiratory acidosis.
3 = Fully compensated respiratory acidosis or non-respiratory basosis.
4 = Partly compensated non-respiratory basosis.
5 = Uncompensated non-respiratory basosis.
6 = Combined non-respiratory basosis and respiratory basosis.
7 = Uncompensated respiratory basosis.
8 = Partly compensated respiratory basosis.
9 = Fully compensated respiratory basosis or non-respiratory acidosis
10 = Partly compensated non-respiratory acidosis.
11 = Uncompensated non-respiratory acidosis.
12 = Combined non-respiratory and respiratory acidosis.

Fig. 5. Schematic Representation of Acid-Base Disturbances in the pH/log pCO$_2$ Coordinate System. (Reproduced from Siggaard-Andersen[25] by courtesy of the author.)

Siggaard-Andersen's chart of the acid-base disturbances in a pH-log pCO_2 coordinate system[25] (Figure 5) summarizes the significance of major deviations of the pCO_2 from the normal state. To demonstrate the interpretation of the chart, respiratory compensation of non-respiratory acidosis represented by Area 10 will be considered. Increased organic acid production leading to accumulation of non-volatile acids will cause a metabolic (non-respiratory) acidosis. While some organic acids, such as lactic acid, can be eliminated by oxidation to CO_2 and H_2O, others must be eliminated by the kidneys, usually a relatively slow process, which may be futher impaired in renal failure. The resulting reduced blood pH acts as respiratory stimulant, and the increased ventilation results in increased excretion of volatile acid (CO_2) and a consequent fall in pCO_2 of the blood. The metabolic acidosis is thus partially compensated by respiratory response, the degree of compensation largely depending on the primary disease. Typically, as illustrated by area 10 of Figure 5, a reduced pCO_2 of 20 to 30 mm. Hg is found with a blood pH of about 7.2 or 7.3.

Respiratory acidosis, characterized by increased blood pCO_2 (as well as increased total CO_2 and blood pH below 7.35), commonly arises from interference with normal pulmonary gaseous exchange, or other obstacles to adequate excretion of CO_2 by the lungs; such as hypoventilation. Typical causes are obstructive emphysema, pulmonary edema, or injury, CO_2 narcosis, anesthetic or other depression of the respiratory center. Respiratory alkalosis is usually the result of prolonged hyperventilation, leading to decreased blood pCO_2 (and decreased total CO_2 and blood pH greater than 7.45). Common causes are encephalitis, hyperpyrexia, mechanical respiration in respiratory paralysis states, the early stage of salicylate poisoning, and certain transitory conditions such as hysterical hyperventilation or the so-called "effort syndrome"[27]. Often, primary changes in one or more of the independent factors such as CO_2 tension are accompanied or closely followed by alterations in the other factors, thus producing mixed acid-base disturbances. The pCO_2 can vary within the limits of approximately 10 to 140 mm. Hg[20]. The lowest values occur as the result of violent hyperventilation, found in anesthesia or as compensatory hyperventilation in marked metabolic acidosis in diabetic coma or renal failure. Very high values result from severe chronic pulmonary insufficiency, acute respiratory failure and CO_2 narcosis[25].

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C. Electrometric Method for pCO_2 in Serum

INTRODUCTION

Of the five practical methods for estimating the pCO_2 (= carbon dioxide tension) of serum, electrometric measurement with the direct pCO_2 electrode offers the important clinical advantages of rapidity and relative simplicity. In 1955 Stow, Baer and Randall[1] first described a CO_2 electrode in which a latex membrane separated the sample from a film of distilled water on the surface of a glass electrode. The CO_2 in the specimen diffused through the rubber into the water until pCO_2 equilibrium was reached. The resulting pH of the water film was found to be related to log pCO_2. Practical improvements and modifications in this system were introduced by Severinghaus and Bradley[2] and by Snell[3].

The procedure described employs the Severinghaus pCO_2 electrode incorporated into a thermostated cell, together with a sensitive pH meter and a circulating thermostat. This equipment can also be used for serum or blood pH determination and pO_2 estimation by addition of a blood pH electrode and an oxygen electrode, and is generally so equipped.

PRINCIPLE

The basic reaction involved in direct pCO_2 measurement with the Severinghaus electrode is

$$CO_2 + H_2O \rightarrow H_2CO_3 \rightarrow H^+ + HCO_3^- \qquad \text{I}$$

The hydrogen ion concentration is a direct function of the partial pressure of carbon dioxide; i.e., $H^+ = a \bullet pCO_2$ or $pH = b \bullet \log pCO_2$ (where a and b are proportionality factors). The actual measurement is carried out in a special glass electrode (arched McInnes type) with a silver chloride electrode as a reference electrode. A thin film of sodium bicarbonate and sodium chloride solution is trapped between the glass electrode surface and a cast *Teflon®* membrane, which is permeable to CO_2 and other gases, but is impermeable to bicarbonate and other ions. Carbon dioxide migrates into the captive electrolyte from the specimen outside of the Teflon membrane in proportion to the sample pCO_2 until equilibrium has been established. The CO_2 molecules dissolve in the bicarbonate solution to form carbonic acid, lowering the pH of the solution. Since the pH decreases one unit for a ten-fold increase in pCO_2, the pH is a linear function of the logarithm of pCO_2, and the latter can be read directly in millimeters from a logarithmic pH meter scale calibrated in pCO_2, once CO_2 equilibrium has been established between the specimen and the captive electrolyte.

The relation is temperature-dependent, and the analysis and calibrations are therefore carried out at a fixed temperature, usually 38° C. Calibration of the electrode-electrometer combination is accomplished with compressed gases of accurately known CO_2 composition (and hence known pCO_2) at the same temperature.

REAGENTS

1. *Carbon Dioxide Compressed Gas, Approximately four Per cent in Oxygen.* A standard CO_2/O_2 mixture containing approximately 4 per cent CO_2 is obtained commercially analyzed within ± 0.02 per cent; or the CO_2 content of the mixture can be determined as outlined subsequently.

2. *Carbon Dioxide Compressed Gas, Approximately eight per cent in Oxygen.* The composition is established within ± 0.02 per cent.

3. *Electrode Solution.* 0.005 M sodium bicarbonate + 0.02 M. sodium chloride. Forty-two mg. anhydrous reagent grade

sodium bicarbonate plus 116 mg. anhydrous reagent grade sodium chloride are dissolved in distilled-demineralized water and the volume adjusted to 100 ml. The solution is stored in a tightly closed borosilicate glass container (not in a polyethylene bottle) to exclude atmospheric CO_2.

4. *Sodium Hydroxide, 0.0200 M.* Exactly 800.2 mg. of reagent grade sodium hydroxide are dissolved in distilled-demineralized water, and the volume is adjusted to 1 l. The weight of NaOH is adjusted appropriately if the solid reagent is assayed at less than 100 per cent NaOH.

SPECIAL APPARATUS

1. pCO_2 Electrode, Radiometer Type E5030 #

2. pH Meter, Radiometer Type PHM27, with Gas Monitor, Type PHA 927 #

3. Circulation Thermostat, Radiometer Type VTS 13 #

Equivalent apparatus for pCO_2 estimation employing the Severinghaus electrode is available from several commercial sources.

PROCEDURE

Collection and Preparation of Specimens

1. Arterial or venous blood can be used; the specimen volume required is too great for capillary blood collection. Venous blood should be drawn with minimum stasis and after the tourniquet has been released.

2. Blood specimens should be placed into small glass tubes and stoppered immediately, and the serum separated from the clot without delay. It can then be preserved in closed micro test tubes for about one hour at 0° C.[4].

3. The blood specimen should be centrifuged only briefly and in a closed container, since up to 8 per cent of the water, and most of the dissolved CO_2, can be lost through the forced draft.

Standardization of the pCO_2 Electrode.

1. The circulation thermostat is turned on, and the equipment is allowed to reach stable temperature conditions at 38° C. The sample temperature adjustment control of the pH meter is set to 38° C.

2. The low setting of the pCO_2 scale of the pH meter is adjusted to 20 mm. Hg with the water-saturated low CO_2 calibration gas flowing through the cell. Then the high setting of the pCO_2 scale is adjusted to the value A with the water-saturated high CO_2 calibration gas flowing through the cell,

$$\text{where } A = 20 \times \frac{pCO_2 \text{ of high } CO_2 \text{ calibration gas}}{pCO_2 \text{ of low } CO_2 \text{ calibration gas}} \qquad \text{II}$$

3. The pCO_2 scale reading is finally adjusted to the true pCO_2 of the high CO_2 calibration gas mixture.

Measurement of the Serum pCO_2

1. The pCO_2 electrode is calibrated at 38° C. and then flushed free of liquid drops with the high CO_2 calibration mixture.

2. The serum sample is introduced

#London Co., Westlake, Ohio.

slowly to displace the gas in the electrode.

3. The pCO_2 of the serum specimen is obtained directly in mm. Hg from the pCO_2 scale of the pH meter when the scale indication has become constant.

4. The pCO_2 electrode is emptied, flushed successively with water, mild detergent solution (e.g., a 0.001 per cent *Sterox SE* solution containing an antifoam agent) and distilled-demineralized water.

Analysis of the CO_2 Calibrating Gas[5].

The pCO_2 of a CO_2 calibrating gas mixture can be determined by equilibrating a 0.0200 M sodium hydroxide solution with the gas mixture and measuring the pH.

At 38° C., the pCO_2 can be calculated as follows:

$$\log pCO_2 = -1.006\,pH + 9.093 \qquad \text{III}$$

CALCULATIONS

Calculation of pCO_2 of a CO_2 Calibration Gas

pCO_2 of a moistened CO_2 gas stream, mm. Hg =

$$\frac{(\text{Barometric Pressure} - pH_2O) \times C}{100} \qquad \text{IV}$$

where pH_2O = vapor pressure of water at ambient temperature, mm. Hg
= 49.7 mm. Hg at 38° C.
where C = Concentration of CO_2 in gas, % V/V

Conversion of Serum pCO_2 from 38° C. to Patient's Temperature[6]

$$pCO_2^{\,t} = \text{antilog}\,[\log pCO_2^{38} - 0.021 \times (38 - t)] \qquad \text{V}$$

where t = patient's temperature, ° C.

RANGE OF NORMAL VALUES

The pCO_2 of arterial plasma or serum varies from 35 to 48 mm. Hg (Mean = 40 ± 2) in adults at sea level[4, 5, 11]. It is slightly lower in infants, and about 3 mm. lower in women than in men[12]. Normal venous plasma or serum pCO_2 varies from 45 to 58 mm. Hg in adults[5, 13].

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The Electrometric Method for Blood pH

JENO E. SZAKACS, M.D., AND ADELBERT CRAMER, M.D.

INTRODUCTION AND PRINCIPLES

THE pH SCALE

Acidity of biologic fluids depends on their hydrogen ion activity. Hydrogen ion activity in blood, an aqueous fluid of great complexity defies strict theoretical analysis. Compared to a conventional scale, however, hydrogen ion activity (aH) in blood is maintained within very narrow limits. As minute changes are of clinical significance, measurements should be made with the greatest accuracy.

A scale for hydrogen ion activity was evolved between 1909 and 1924 by Sørensen[26] and Sørensen and Linderstrøm-Lang[27]. Activity is defined as the product of the molar concentration of hydrogen ions and the activity coefficient (f).

$$aH = fH \cdot mH$$

In water, aH is limited by the possible molar concentrations of acids or bases which are rarely higher than 10 mol. per l. As the values for f are less than unity, in most cases, the value of aH will be a small fraction of one. Sørensen proposed to express these fractions by their negative logarithm and assigned the notation paH, which become simplified to pH.

$$pH = -\log aH$$

Electrometric pH determinations are based on measurements of the potential difference (e.m.f.) developed between two electrodes immersed in a solution. If certain requirements are met by the electrode cell assembly, the e.m.f. of the cell serves as a formal measure of hydrogen ion activity. One of the electrodes needs to be selectively sensitive and reversible to hydrogen ions. The other electrode serves as reference, and it is required to produce a standard reference potential against the solution. The ultimate standard for reversible hydrogen ion sensitive electrodes is the H_2 gas electrode. The origin of the potential is easily understood on the basis of the electrode reaction of the H_2gas electrode:

$$\tfrac{1}{2}\, H_2(\text{gas}) = H^+ + e^-$$

In its simplest form, the H_2gas electrode is a platinated-platinum wire or foil partially immersed in a buffer solution and exposed to a stream of pure H_2 gas. When the pH of the buffer solution is unity and the H_2 gas saturates both buffer and electrode at one atmosphere pressure, the electrode is referred to as the "Standard Hydrogen Electrode." The electrode potential (E°) of the standard electrode is by definition equated to zero potential at all temperatures. A scale with its zero so defined is referred to as the "Hydrogen Scale."

If the buffer solution of unit pH is replaced by a test solution in the standard H_2 gas electrode, the potential difference (E) developed becomes a formal measure of pH. According to the osmotic theory of Nernst, the quantitative relationship between e.m.f. and pH for the half cell is expressed by the following equation:

$$E = E^\circ - \frac{2.303\ RT}{F}\ \log.\ aH$$

E° by definition equals zero

$$E/pH\ unit = \frac{-2.303\ RT}{F}$$

R is the gas constant, T is absolute temperature, and F is the faraday.

The pH scale, theoretically at least, could be defined and fixed on the basis of electrode response of a H_2 gas electrode. In practice, it is necessary to make measurement on complete cells. The origin of differences in pH scales find their explanations in this necessity. In the complete cell, secondary reactions have a definite influence on electrode potentials. The most important of these factors are the diffusion potential developed at liquid junctions between solutions of different electrolyte composition (salt bridge), and the influence of the activity coefficient and ionic strength of the chloride ion. On the basis of extensive studies, Bates and Guggenheim[2] employed cells without liquid junction and proposed a single convention for Cl-ion activity. pH values were assigned accordingly to a series of primary standards that now serve to fix the NBS pH scale[3]. The assigned values are reproduced in Table I and are theoretically significant to ±0.003 pH unit. This scale gives 0.04 pH unit higher values than Sørensen's scale.

The type of electrode cell assemblies might vary according to the scope of the measurement. Böttger[8], in the earliest successful attempt to measure acidity versus titratable acid, employed the H_2 gas electrode. The H_2 gas electrode shows perfect linearity in response to pH, but it is not versatile and not suitable for determinations where pH depends on dissolved gasses (e.g. CO_2) in the test solution. For its linearity of response it is employed to standardize glass electrodes and primary buffers necessary for electrometric pH determinations.

The Operational pH

This is determined with electrode assemblies connecting the two half cells through a liquid junction. The cell might be written as follows:

electrode reversible to hydrogen ion	buffer or unknown sol.	bridge electrolyte (sat. KCl)	reference electrode

The double line represents the liquid junction.

The experimental pH value of an unknown solution is obtained through two measurements. First, the e.m.f. (E) of the cell with the unknown is determined, and then the unknown is replaced by a primary standard buffer, and the e.m.f. (Es) is determined. The pH of the unknown is related to the e.m.f. measurements as described by the equation

$$(2) \quad pH = pH_s \frac{E - E_s}{(RT \ln 10)/F}$$

where $\frac{RT \ln 10}{F}$ = Nernst's constant in log base 10.

pH_s = hydrogen ion activity of the standard

R = Gas constant (8.3149 joules/degree/mole.)

T = Temperature in degrees Kelvin.

F = The faraday (96,493.1 coulombs)

ln 10 = 2.3026

Modern pH meters are calibrated both in millivolts and pH units. Their potentiometric circuit allows for "standardization" by fixing the meter scale to the assigned value of the primary standard. The pH value of the unknown is simply read from a scale. However, the determination is based on the difference of pH activity between test and standard solution. This type of measurement does not require an accurate determination of the standard potential of the cell. The reference potential needs to remain constant only during the actual measurement and linearity of response from the hydrogen sensitive electrode is expected only within a narrow range of the pH scale. Still, the assembly can provide great precision[4, 5, 21].

THE GLASS ELECTRODE

Electrodes reversible to hydrogen ions include the quinhydrone, the antimony-antimony oxide, and the glass electrode, besides the previously-mentioned hydrogen electrode. Bates recently reviewed the advantages and disadvantages of these electrodes[2]. The glass electrode is most versatile, also ideally suitable for biologic measurements[14]. It consists of a pH sensitive membrane of hygroscopic glass exhibiting relatively low electrical resistance and can vary in chemical composition. One of the best known glasses was described by McInnes and Dole (Corning 015)[15], and it has a formulation of 72.2 mole per cent SiO_2, 21.4 mole per cent Na_2O and 6.4 mole per cent CaO. In more modern glasses, lithium is substituted for the Na to reduce the alkali error of the electrode. Table III, reproducing characteristics of some commercial electrodes, is from the paper of Bates previously mentioned[2]. Two general types of glass electrodes are now employed, the bulb type and the capillary type. In each type, one surface of the glass membrane is in contact with a buffer of constant hydrogen ion activity (0.1 m HCl) and contains a reference electrode, usually silver-silver chloride. The other surface is exposed intermittently to the test solutions and standard buffers. This surface, however, is to be conditioned (about 24^h) by exposing it to water before the electrode is activated so it will become truly reversible to hydrogen ion activity. The glass surface, when properly hydrated, swells, and some of its alkali metal ions become dislodged from the interstices of the silicon-oxygen lattice by the hydrogen ions of water[17, 10]. The hydrated surface of the glass electrode readily exchanges hydrogen ions with the solution, and a reversible equilibrium develops. Transfer of ions through the junctional surface of the glass electrode is thought to be responsible for the development of the electrode potential or pH response. Change in this potential is measured across the glass membrane as added voltage to the standard potential of the silver-silver chloride reference electrode. The glass electrode can be considered a concentration cell, and it is remarkably free from interference by oxidation-reduction potential and ionic interference in a wide range of the pH scale, including Na ions[10]. It has, however, a limited life as its hydrogen ion response depends on partial leaching of its alkali ions from the glass surface. Its pH response might be reduced by protein "poisoning," etching, or such mechanical injury to the surface as occurs when a conditioned (hydrated) electrode is exposed repeatedly to drying, or more obviously when the electrode is scratched. For these reasons, and to quantitate the deviation from ideal response of the glass electrode in the acid and alkali extremes of the pH scale (see figure 2), Perley[18] advocated periodic standardization of electrodes against the H_2 gas electrode. A modern hydrogen electrode cell is described by Sibbald and Matsuyama[23], and it is eminently suitable for standardization of buffers and electrodes.

THE REFERENCE ELECTRODE

The reference electrode is commonly a calomel electrode (see Figure 1). Calomel electrodes are formed by opposing to liquid mercury in a glass tube a mixture of calomel powder ($Hg_2 Cl_2$) and mercury. The tube is then suspended in an outer vessel filled with a solution of KCl, usually concentrated and provided with one of many devices to form a reproducible liquid junction, such as porous plugs, sleeves, wicks, etc. The saturated calomel electrode is the source of a constant reference potential. Sibbald and Matsuyama reported standard deviation of ± 0.14 mv. during a two week period in the electrode potential of a calomel electrode measured with their (modified) hydrogen cell assembly. The average electrode potential given by these authors was 243.33 mv. at 25° C. (Beckman No. 39071 saturated calomel electrode with carborundum frit junction[23]). The geometry of the area of junction between the KCl solution and test solution is of great significance. On it depends the reproducibilty of the liquid junction potential. Increasing concentrations of KCl serve to reduce the junction potentials[4].

TEMPERATURE INFLUENCE

Hydrogen ion activity is dependent upon temperature. The absolute temperature (T) is one of the three factors in Nernst's constant. For buffers the magnitude of variation in pH at different temperatures is evident from Table I. Two independent temperature coefficients are in play, that of the electrode cell and that of the buffer. Precise measurement, therefore, requires

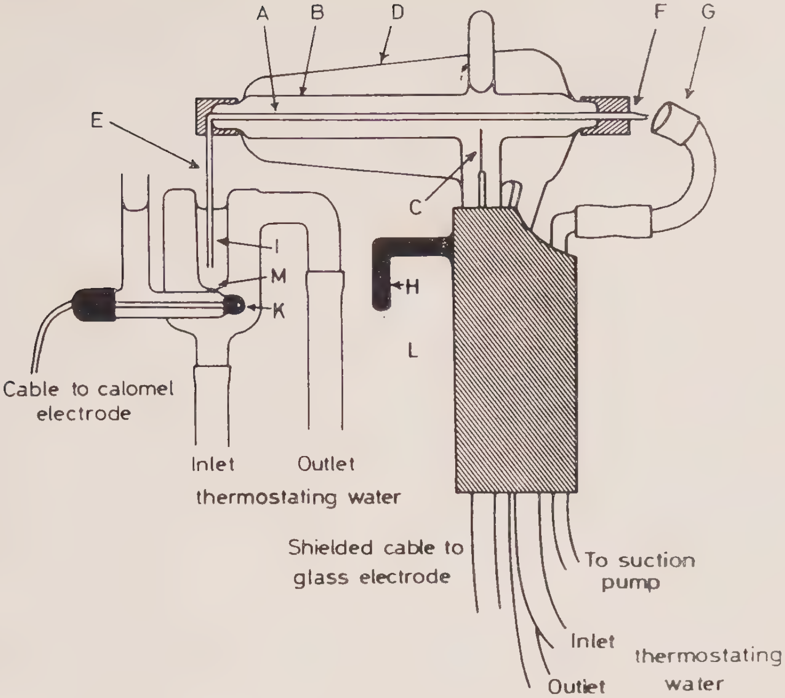

Fig. 1. Thermostated capillary glass electrode and calomel electrode (Siggaard-Andersen, Engel, Jørgensen and Astrup, 1960) made by Radiometer, Coppenhage.

(a) Capillary glass electrode, surrounded by a buffer in tube (b) (c) The Ag/AgCl reference electrode in the buffer fluid. (d) The outer envelope of the thermostating water jacket. (e) Polyethylene tip that serves to fill the electrode and to form at its tip the liquid junction with the calomel electrode (k) (m) A porous disc which connects the salt bridge (l) to the calomel electrode (k).

temperature equilibrium of electrodes, bridge solution, standards, and unknown. This can be obtained by well regulated constant temperature baths. Robinson and Pimblett[19] found that it takes twenty-four hours for a glass electrode, and more than forty-eight hours for a calomel electrode, to come to thermal stability. Thermostating should include the calomel reference electrode, and it is recommended to be accurate to ± 0.05° C.

Whole blood shows considerable change in pH with temperature changes. Rosenthal[20] found experimentally the temperature coefficient for whole blood to be:

$$\Delta \text{pH} = -0.0146 \Delta \text{T}$$

where Δ pH is the difference in pH and Δ T the difference in temperature.

The temperature of blood pH measurement is of great significance. The normal blood pH apparently changes with body temperature. Measurements have to be made, however, at a convenient temperature, as temperature equilibrium of the electrode assembly cannot be obtained in a short period of time. Generally 38° C. is selected,

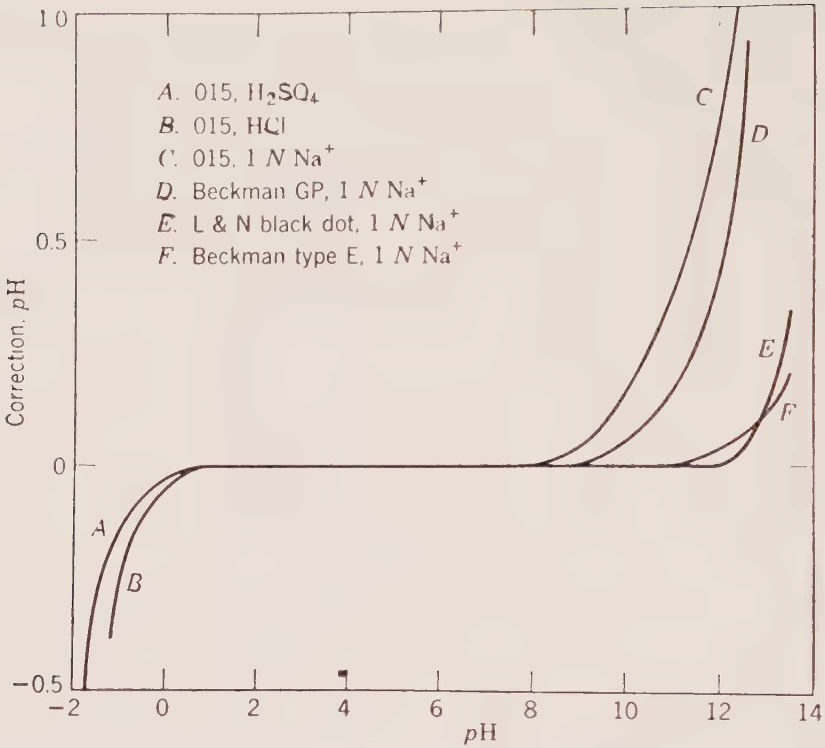

Fig. 2. Deviation from ideal response of the glass electrode. Comparison with the H_2gas electrode shows the acid and alkali corrections at 25° C. From Bates, R. G., reference 4.

regardless of the patient's possible fever or hypothermia.

The pH Meter

The pH meter serves to detect and quantitate the potential generated by the pH cell assembly. A standard electrode potential is generated by both the inner silver-silver chloride reference electrode and the reference calomel electrode. The potential difference of the two reference electrodes is 44 m volts when they are connected through saturated KC1.* The electrode response of the glass electrode is measured as added voltage with positive increments for each pH unit below seven, and negative increments for each pH unit above seven on the pH scale (see Figure 3). The increment itself is given by Nernst's coefficient and the efficiency of the glass electrode, the latter being about 98 per cent.

$$\text{Nernst's coefficient, } \Delta E = \frac{-2.303\ RT}{F}$$

where Δ E, the increment of potential per pH unit is temperature dependent and gives a straight line with a slope of −61.7 mv. per pH unit at 38° C. or −58.2 mv. per pH unit at 20° C. with

---

*Ag, AgC1, 0.1N HC1/KC1 sat., calomel, Hg

Ag, AgC1, 0.1N HC1/sat. KC1 electrode potential at 25° C.	−0.1992
Sat. KC1, Calomel, Hg electrode potential at 25° C.	+0.2433
Net cell potential	0.0441

pH7 at −44 m volt. Thus the temperature coefficient has little influence on measurements close to pH 7 and increasing influence on measurements on either extreme of the pH scale.

The internal resistance of the electrode cell assembly is almost entirely due to the resistance of the glass electrode. It amounts to 100 to 1000 megohms. In order to detect the pH response "hidden behind" the resistance of the glass membrane, the pH meters are equipped to provide high input impedance in the order of 100,000 megohms. This means that the input signals need to be amplified to a very high degree, and heavy shielding and insulation are needed.

Precision pH meters may be of two general types. One is a null balanced electronic potentiometer provided with a standard cell producing a measured bucking potential that opposes that of the electrode cell. This type of equipment usually employs a vibrating reed to convert the direct current signal to alternate current suitable for drift free amplification. The second type of meter (see Figure 4) is slightly more simple but somewhat less accurate[9]. It employs a "chopper" or modulator to convert the d.c. signal to a.c. suitable for amplification. Intermittently the chopper is in metallic contact with the glass electrode and draws a small percentage of the electrode voltage. The amplified current so obtained is used to drive a direct-reading volt meter, and through a resistor it is connected to the reference calomel electrode. The meter voltage

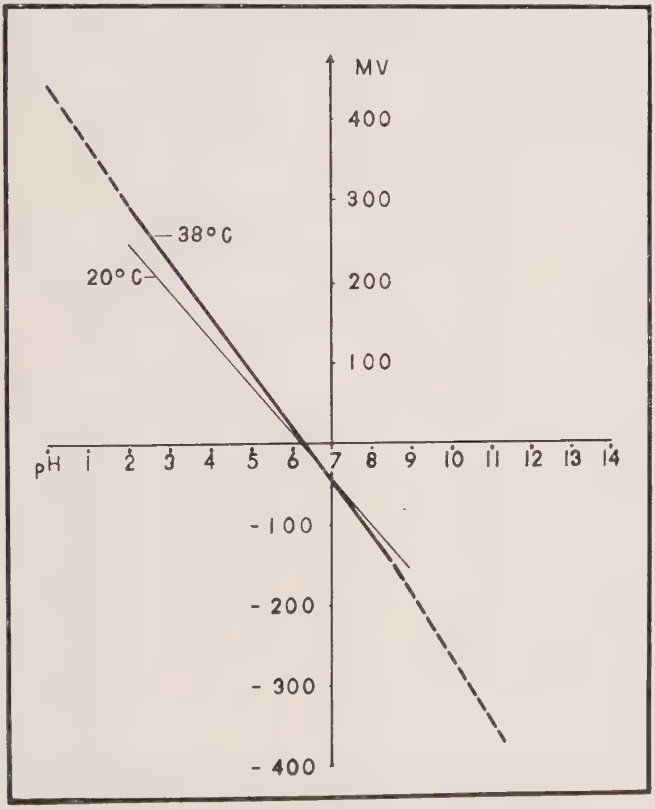

Fig. 3. Typical pH response of the glass electrode.

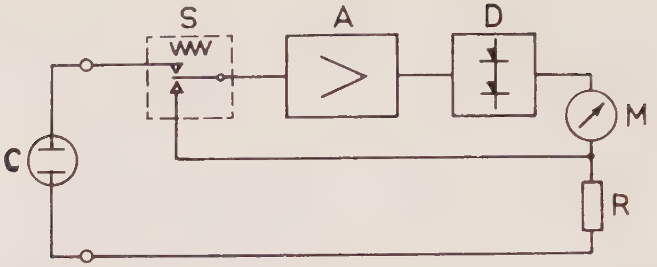

Fig. 4. Simplified diagram of a chopper type direct reading pH meter Radiometer pHM 25 or 27. From N. Brems, reference 9.

C — Symbolizes the electrodes and sample solution
S — The chopper, a relay-like switch
A — Amplifier
D — Phase sensitive demodulator
M — Volt meter
R — Resistor

rises to indicate the electrode cell potential. The resistance is crucial in calibrating a pH meter of this type.

The electro-technical progress in precision pH meters has outstripped progress in theoretical and practical definition of the pH scale itself. The Beckman research pH meter, model 101900, and the Radiometer pH M-4 are both capable of discriminating 60 microvolts or 0.001 pH units.

The Sample

Arterial whole blood is the sample of choice for the determination of blood pH. Venous blood might show considerable variation not directly referable to arterial pH, unless the patient is fasting and is resting recumbent. Venous pH depends on the blood flow and the metabolism of the region drained. It is reportedly from 0.0 to −0.03 pH lower than arterial pH in a resting extremity[12]. Arterialized capillary blood is well suited for pH determinations with capillary electrodes that require but 50 microliter or less of blood per determination. The ear lobe or the finger tip are recommended as convenient sites for collection of blood. In our experience the ear lobe proved to be useless in severely ill or comatose patients, while the hand was found more easily manageable. The patient's hand is placed in hot water (about 45° C.) for ten minutes, then dried, one of the fingers wiped with an alcohol sponge, again dried, and then punctured. A number 11 BD scalpel blade produced a free flow of arterialized capillary blood. The first drop of blood is discarded. The subsequent drops are collected. Two virtually anaerobic collection methods have been recently perfected; the very elegant method of Siggaard-Andersen[24] and Astrup 1959[1] using capillary tubes with dried heparin and fluoride, and the method of Gambino[11] employing a tuberculin syringe, heparinized and fitted with a rubber cup. Either method works well on ambulatory patients, while the sick patient with reduced peripheral circulation causes difficulties. Clotting occurs in the capillary tube method due to slow collection and delay in mixing of the sample. The syringe method seems to need less dexterity, and it is not so critical as far as clotting is concerned. Four tenths to five tenths ml. of blood is collected in the heparinized TB syringe; then, 0.05 to 0.1 ml. of mercury is aspirated to form a seal and later to facilitate mixing. The syringe is capped and placed in a glass of ice water until it is transferred to the laboratory. There should be as little delay as possible before measurements, even though the pH of chilled blood remains unchanged for up to four hours.

Considerable error can be introduced if blood is allowed to run over the skin surface, or if air bubbles or foam are allowed to form in the capillary or syringe. With correct technique, without interruption of the surface tension barrier of the drops of blood, a virtually anaerobic collection is obtained, and the use of neutralized paraffin oil can be avoided[13]. Before measurement, the blood sample is thoroughly mixed, as redistribution of CO_2 upon cooling occurs from plasma to red cells[25].

Plasma separated anaerobically at 38° C might be substituted for whole blood[12]. Blood pH is altered by clotting. Therefore, serum cannot be used in lieu of blood or plasma for pH determination.

STANDARD SOLUTIONS

Considerable effort was made to obtain an internationally acceptable series of primary standards[6, 16, 21]. Table I lists the recommended National Bureau of Standards pH values for seven buffers at different temperatures[3].

TABLE I REVISED VALUES FOR pH MEASUREMENT[*]
(RECOMMENDED pH, FOR SEVEN STANDARD BUFFERS)

	A	B	C	D	E	F	G
t	TETROXALATE	TARTRATE	PHTHALATE	PHOSPHATE	PHOSPHATE	BORAX	CALCIUM HYDROXIDE
°C							
0	1.666	....	4.003	6.984	7.534	9.464	13.423
5	1.668	....	3.999	6.951	7.500	9.395	13.207
10	1.670	....	3.998	6.923	7.472	9.332	13.003
15	1.672	....	3.999	6.900	7.448	9.276	12.810
20	1.675	....	4.002	6.881	7.429	9.225	12.627
25	1.679	3.557	4.008	6.865	7.413	9.180	12.454
30	1.683	3.552	4.015	6.853	7.400	9.139	12.289
35	1.688	3.549	4.024	6.844	7.389	9.102	12.133
38	1.691	3.548	4.030	6.840	7.384	9.081	12.043
40	1.694	3.547	4.035	6.838	7.380	9.068	11.984
45	1.700	3.547	4.047	6.834	7.373	9.038	11.841
50	1.707	3.549	4.060	6.833	7.367	9.011	11.705
55	1.715	3.554	4.075	6.834	....	8.985	11.574
60	1.723	3.560	4.091	6.836	....	8.962	11.449
70	1.743	3.580	4.126	6.845	....	8.921	.....
80	1.766	3.609	4.164	6.859	....	8.885	.....
90	1.792	3.650	4.205	6.877	....	8.850	.....
95	1.806	3.674	4.227	6.886	....	8.833	.....

[*] From R. G. Bates, Journal of Research N. B. S., 66:179-184.

The composition of the standard buffer solutions, as recommended by Bates, is listed in Table II. Carbon dioxide free water is to be used for the preparation of the phosphate and borate buffers. The buffer substances are available as certified standard samples from the National Bureau of Standards. Commerical buffers, such as Beckman's and Radiometer's, are available and are made to National-Bureau-of-Standards' specifications. The standard buffers are to be handled as primary standards. They should be bottled in glass as plastic is permeable to CO_2. They should be placed in small bottles and opened only one at a time, avoiding exposure to air as much as possible. A small portion of the buffer should be poured into a beaker for immediate use. The excess left in the beaker should be discarded.

TABLE II COMPOSITIONS OF SEVEN STANDARD BUFFER SOLUTIONS*
(Weight of Buffer Substance (in air) per Liter of Buffer Solution at 25° C.)

SOLUTION		BUFFER SUBSTANCE	WEIGHT IN AIR
			g
A, Tetroxalate,	0.05 M.	$KH_3(C_2O_4)_2 \cdot 2H_2O$	12.61
B, Tartrate, about	0.034 M.	$KHC_4H_4O_6$	Saturated at 25° C
C, Phthalate,	0.05 M.	$KHC_8H_4O_4$	10.12
D, Phosphate,	0.025 M.	KH_2PO_4	3.39
	0.025 M.	Na_2HPO_4	3.53
E, Phosphate,	0.008695 M.	KH_2PO_4	1.179
	0.03043 M.	Na_2HPO_4	4.30
F, Borax,	0.01 M.	$Na_2B_4O_7 \cdot 10H_2O$	3.80
G, Calcium hydroxide,	0.0203 M.	$Ca(OH)_2$	(Saturated at 25° C.)

*Same source as Table I.

At least two buffers are needed to check linearity of response of the glass electrode. Buffer "D" of Table I, the equimolal phosphate of pH 6.840 at 38° C. and buffer "E," a phosphate buffer of pH 7.384 at 38° C., are recommended for this purpose. For the most precise measurements, interpolation of each unknown between the values of two primary standards is necessary. For routine clinical work the linearity of response is checked only intermittently, once for each series of determinations. Buffer "E" pH 7.384 is employed as a primary standard for blood pH determination, as its pH value is closest to that of blood.

The buffers are usually stored in a refrigerator. They should be brought to the temperature of the pH cell assembly prior to measurement. Unless temperature equilibrium of the cell is maintained, serious errors might be introduced. Of the different types of electrodes, the capillary glass electrodes are least sensitive to temperature change, as apparently the small amount of buffer introduced assumes the electrode temperature rapidly.

The same precautions are to be taken with such other solutions as might be recommended by the manufacturer for cleansing the electrodes between measurements. Isotonic saline (0.85 per cent) is used to rinse the electrode before and after blood is introduced. Rinsing of the electrodes with 0.1 N HCl and with pepsin reduces the effects of protein "poisoning." The glass electrodes are finally rinsed and stored in distilled water.

A saturated solution of KCl is needed to replenish the calomel electrode and the salt bridge. To assure saturation at 38° C., a few crystals of KCl are added to the electrolyte in the reservoir of the calomel electrode.

TABLE III CHARACTERISTICS OF SOME COMMERCIAL ELECTRODE GLASSES AT 25°*

(Data taken in part from Simon and Wegmann)

ELECTRODE	COMPOSITION OF GLASS	RESISTANCE (MEGOHMS)	CORRECTION** IN 0.1 M NaOH	(pH UNITS) 1 M NaOH
Beckman E-2	SiO_2, BaO, Li_2O	375	0.03	0.17
Beckman General Purpose	SiO_2, BaO, Li_2O	150	0.43	1.4
Beckman Amber	SiO_2, BaO, Li_2O	550	0.02	0.17
Cambridge Alki	——	400	——	0.15
Corning 015	SiO_2, CaO, Na_2O	90	1.0	2.5
Doran Alkacid M_{499}	——	300	0.02	0.10
Electronic Instruments GHS	SiO_2, Li_2O, Cs_2O	200	0.02	0.15
Ingold UN	SiO_2 Li_2O	30	0.70	1.9
Leeds and Northrup "Black Dot"	SiO_2, La_2O_3, Li_2O	70	0.02	0.25
Metrohm H	SiO_2, BaO, Li_2O	1400	0.08	0.15
Metrohm X	SiO_2, CaO, Li_2O	100	0.90	2.2
Metrohm U	SiO_2, BaO, Li_2O	500	0.08	0.26

*From R. G. Bates[5].

**To be added to apparent pH value.

SPECIAL APPARATUS AND PROCEDURE

The research pH meter of Beckman Instruments, Inc., and the PHM-4 model meter of the Radiometer Co. are provided with capillary glass electrodes and calomel electrodes for anaerobic measurement of blood pH. The principles involved in measurements of electrode potentials were already mentioned. The simplified circuit diagram of the Beckman Research pH Meter[7] serves to illustrate the electronic adjustments that are made to obtain precision (see Figure 5).

The first adjustments to be made serve to *calibrate* the instrument against a standard potential furnished by a cell contained within the bridge circuit. For this procedure the scale is selected to read −MV x 100, and then the READ control is set to $\frac{1}{100}$ of the standard voltage. The CALIBRATE control is then used to balance the null meter.

The second adjustments serve to *standardize* the electrode system. A buffer of known pH is aspirated into the electrode, for example buffer "E," if blood pH measurement is contemplated. The TEMP control is adjusted to the actual temperature of the cell assembly; the READ control is set to the exact pH value of the buffer at the given temperature; and then the STANDARDIZE control is adjusted to balance the

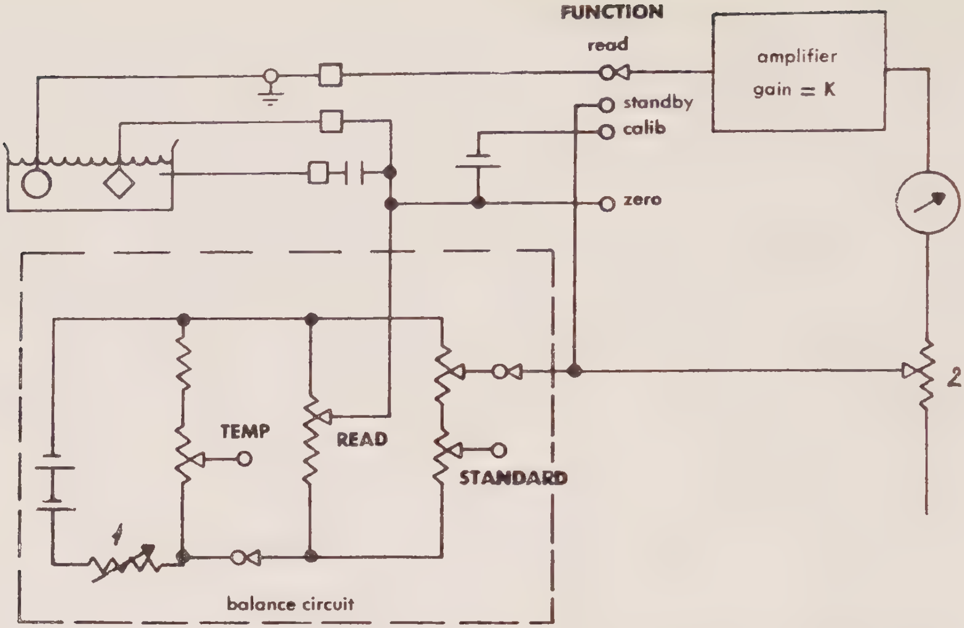

Fig. 5. Simplified circuit diagram of the Beckman Research pH Meter, from Beckman Instructions 1226-A, Fullerton, California.

1. Calibrate control.
2. Sensitivity control.

null meter. This standardization compensates for all the various potentials in the electrode system.

Subsequent aspiration of an unknown solution into the capillary electrode will produce a potential proportional to its pH. This potential, then, is balanced out by the READ control, and it is read directly in pH units. Detailed instructions are given by the manufacturer on procedure to be followed. It might be necessary to make one or two preliminary readings prior to the final one, if the most precise results are sought.

Upon completion of the measurements the capillary electrode is aspirated free of blood and washed with saline, 0.1N HC1 and distilled water. Glass electrodes are maintained under water or filled with water. Effects of protein coating are minimized by filling the electrode with a solution of pepsin overnight.

DISCUSSION

The described electrodes and pH meters are suited to measure blood pH with a standard deviation of about 0.006 pH unit[1]. Errors might be introduced by improper handling of the blood specimen. These errors can be controlled if the procedures outlined are strictly adhered to, if that is, a virtually anaerobic collection is made and if, the blood is immediately chilled and completely mixed prior to measurement.

Errors due to change in linearity or protein "poisoning" of the glass electrode are easily detected upon standardization against two buffers of known pH, and can be avoided to a large extent, by proper cleansing of the electrodes.

Errors might be due to lack of thermal equilibrium of the electrode assembly. Thermostating of the entire cell with a rigid temperature control at 38° C. ± 0.05° C. solves this problem effectively.

The care for the porous junction of the calomel electrode includes cleaning all coagulated blood away immediately after measurements and maintaining a free flow of the electrolyte solution by refilling the electrode with saturated KC1 to a higher level than that of the fluid outside.

The precision of the measurements depends finally on the precision of the primary standard buffers used. The most meticulous care is needed in the handling of these solutions.

Résumé of Clinical Interpretations

Arterial blood pH is one of the parameters of acid base equilibrium. Blood pH is regulated by the available buffer base and by the partial pressure of CO$_2$. The extreme limits[25] of the "respiratory pH" dependent upon the PCO$_2$ are 7.782 and 7.054 in blood with 15 grams per 100 ml. of hemoglobin. The values correspond to 10 and 140 mm. Hg PCO$_2$, respectively. The extreme changes of pH due to deficit or excess of 30 mEq. of buffer base are 6.829 and 7.741, respectively. This is referred to as "non-respiratory pH." The pH of arterial blood at 38° C. is the arithmetic mean of the respiratory and non-resipratory pH.

Normal values using the NBS scale for arterial or arterialized capillary blood are given by Siggaard-Andersen as:

Man	7.360-7.420	mean 7.390
Woman	7.376-7.420	mean 7.398

The patient is said to be acidotic if the arterial blood pH is below 7.350, or alkalotic if it is above 7.450.

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The Chemical Measurement of Total Base in Serum

HANS N. NAUMANN, M.D.

A. General Considerations

INTRODUCTION

The measurement of serum total base by chemical means was of considerable interest in the 1920s, when serum electrolytes were studied intensively by several groups of investigators[1, 3, 8, 10, 11, 12, 20, 21, 23, 24]. At that time, the principal anions, chlorides and bicarbonates, could be determined with reasonable ease and accuracy, while determinations of the principal cations, sodium and potassium, were difficult and laborious. When, therefore, Stadie and Ross[16] introduced a relatively simple method for determination of serum total base, i.e., Na + K + Ca + Mg, adapted after Fiske's[4] earlier procedure for urinary cations, this at once provided an essential tool for research of electrolyte metabolism. Investigation of the acid-base equilibrium was made possible after the determination of anions was implemented to include protein-bound base[8, 23, 24], phosphates[5] and lactate[2, 3], main component of organic acids. After various modifications[13] the original Stadie-Ross method has been considerably improved and simplified by Hald[7] in 1933, and since then the Hald modification for serum total base has been adopted as a standard of reference.

Heating of serum with sulfuric acid converts the cations, sodium and potassium, calcium and magnesium, into sulfates, and interfering phosphates are bound as insoluble iron phosphate by addition of iron sulfate (Fig. 1). After igniting organic compounds by dry-ashing, the sulfates are dissolved and precipitated by benzidine as benzidine sulfate, which is dissolved in hot water and directly titrated with sodium hydroxide without interference by the very slightly basic benzidine moiety.

NORMAL VALUES

Table I gives a compilation of earlier and more recent values of serum total base, individual cations, anions, and serum total acid. There has been a tendency of decreasing values over the years, particularly of sodium, chlorides, bicarbonates, and total base itself.

The latest and most accurate normal total base values are those of Sackner and Sunderman[14], which were deter-

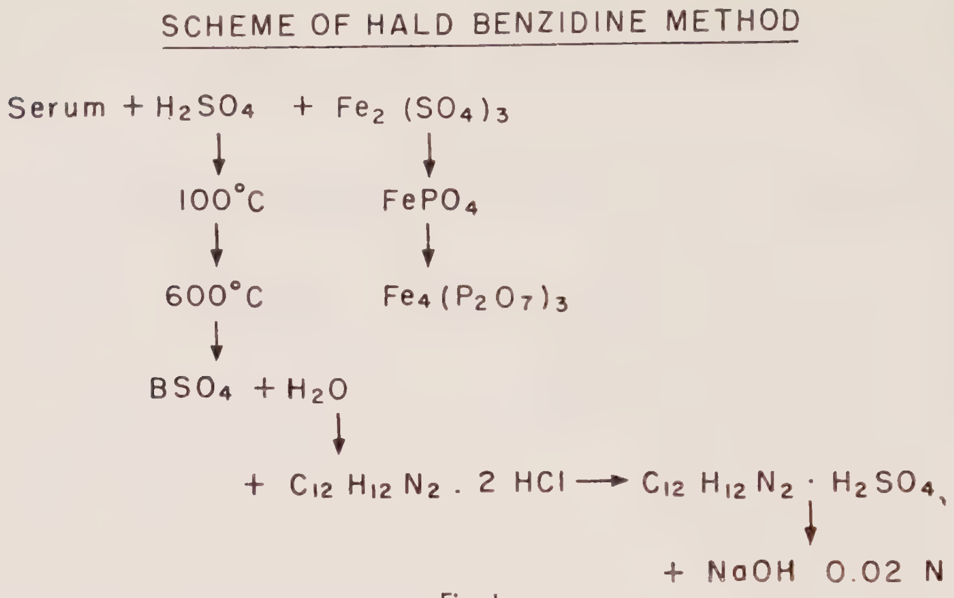
SCHEME OF HALD BENZIDINE METHOD
Serum + H2SO4 + Fe2 (SO4)3
100°C
FePO4
600°C
Fe4 (P2O7)3
BSO4 + H2O
+ C12 H12 N2 . 2 HCl → C12 H12 N2 · H2SO4
+ NaOH 0.02 N

Fig. 1

mined by four methods: conductometry, benzidine method, chemical determination of cations, and flame photometry plus chemical determination. The average normal value calculated from the figures of each of the four methods was 145 mEq./L. with an average standard deviation of ± 2.2. It may be noteworthy that the values of Peters and Van Slyke[13] with 155 mEq./L. of total base dating back to 1931 continue to be used as standards, for example, by Gamble[6], in 1950, with slight changes and more recently by Weisberg[26], while Singer[15] gave a value of 150.

In this connection, attention should be called to the important observations of Kelsey and Leinbach[9] that serum total base in children under twelve years of age was found considerably

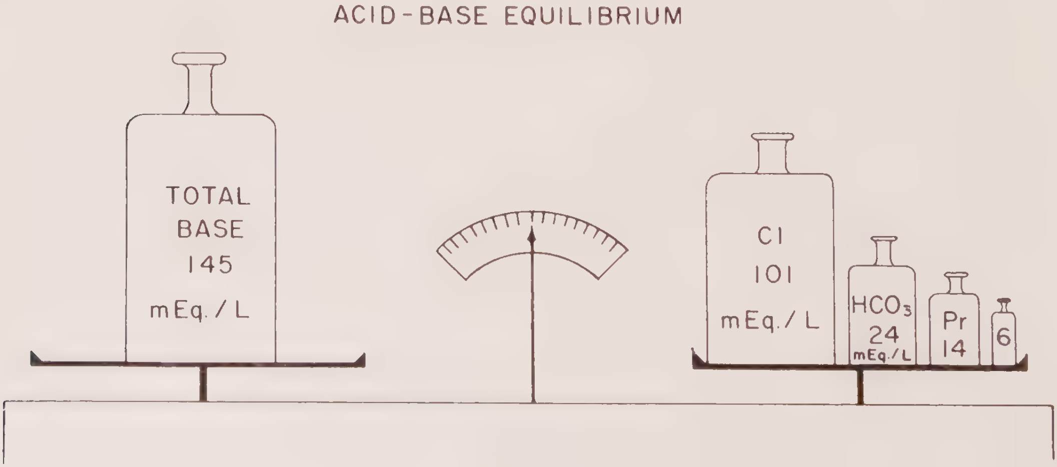
ACID-BASE EQUILIBRIUM

Fig. 2

higher than in adults and the equally important finding of Walaas and Walaas[25] that food intake had a measurable effect on serum total base.

CONTRADICTORY FINDINGS IN ACID-BASE-EQUILIBRIUM

A perplexing problem has been posed by observations of *sera* from normal, as well as diseased individuals where acids and bases were not in equilibrium. Since it must be postulated that, in a neutral fluid such as serum or plasma, all acids are neutralized by all bases, it follows that total base equivalents must equal the sum of acid equivalents (Fig. 2). Accepting Sackner and Sunderman's value of 145 mEq./L. as seen on the left side of the diagram in Figure 2, we find that on the right side of the diagram, 101 for Cl, 24 for HCO_3, 14 for protein and 6 for the remaining anions, phosphates, sulfates, and organic acids add up to 145 as expected. Generally, with adequate methodology, experimental proof has not been lacking except in early investigations (Table I). However, differences up to 8 per cent, generally exceeding the total base value, have been obtained by careful analysts. For instance, Hald[7] found in a series of twelve serum analyses on ten normal individuals 3 *sera*, in which total base was less than total acid by 8.4, 8.2 and 5.4 mEq./L., respectively. Similarly, Peters, Wakeman, Eisenman, and Lee[11] obtained fourteen deficits of total base

Table I Normal Values for Total Base, Total Acid, Cations and Anions from the Literature (mEq/L).

	Cations				Total	Anions						Total
Authors	*Na*	*K*	*Ca*	*Mg*	Base	*Cl*	HCO_3	*Prot.***	PO_4	SO_4	*Org. A.*	*Acid*
Peters *et al.* 1926	—	—	—	--	156	104	26	11	2	—	—	143
Darrow-Hartmann 1929	—	—	—	—	151	100	25	15	2	—	2	145*
Atchley-Benedict 1930	—	—	—	—	152	104	29	18	2	—	—	152*
Peters-Van Slyke 1931	142	5	5	2	155	103	28	16	2	6		155
Sunderman 1931	135	4	5	2	146	101^{22}	24^{22}	14^{22}	7^{22}			146^{22}
Hald 1933	135	5	5	2	146*	103	27	17	—	—	—	147
Gamble 1950	142	5	5	2	155	103	27	16	2	1	6	155
Sackner-Sunderman 1958	133	5	5	2	145	101^{22}	24^{22}	14^{22}	7^{22}			146^{22}
Singer in *Altman-Dittmer Biological Handbook* 1961	139	4	5	2	150	103	26	16	5			150

*Because fractions have been rounded to the nearest whole figure, summations may differ by 1 in the last digit.

**Partly calculated by formula of Van Slyke *et al.*, 1928[24].

ranging from 0.8 to 12.7 mEq./L., and Atchley-Benedict[1] found thirteen base deficits up to 5.8 mEq./L. in 60 analyses.

Seeking an explanation, Hald[7] ruled out gross technical errors and presence of unaccounted but negligible bases such as NH_4, rare metals, and organic bases, and she assumed that the acid side of the balance was at fault. In this connection it should be noted that determinations necessary for a total acid-base equilibrium study rest all on well-founded analytical procedures, with the exception of protein equivalents and organic acids. Proteins, as amphoteric electrolytes at the slightly alkaline pH of serum or plasma, react as weak acids which bind base, and this base-binding capacity is calculated from concentrations of total protein, or of albumin and globulin separately, by one of three different formulas derived by Van Slyke and collaborators[8, 23, 24], or by a formula of Darrow and Hartmann[3]. In Table II an attempt was made to obtain an idea of the normal base-binding value of protein by deducting all other anion values from the total base figure and to compare this deduction with values calculated by each of the four available formulas from average normal serum proteins. It will be noted that the first two columns with higher total base figures by the Stadie-Ross method yield greater protein base values, which agree with those calculated by formula 1, 2 or 4. The lower protein base values of columns 3 and 5, by a modified Stadie-Ross method and averages by four different methods, respectively, agree with the calculated value of formula 3. The low value by Hald in column 4 may be explained by a slightly high HCO_3 and Cl value, increasing the sum of anions and thereby decreasing the deduction figure. On the other hand, the total base value in column 6 appears too high, and PO_4 + SO_4 + organic acids appear too low. Even here, however, a protein value of 16 mEq. per L. would still

TABLE II COMPARISON OF PROTEIN-BOUND BASE FROM NORMAL CATION-ANION DIFFERENCE AND CALCULATED BY VARIOUS FORMULAS*

	1 DARROW-HARTMANN[3] 1929	2 PETERS-VAN SLYKE[13] 1931	3 SUNDERMAN[17] 1931	4 HALD[7] 1933	5 SACKNER-SUNDERMAN[14] 1958	6 SINGER IN BIOL. HNDBK.[19] 1961
Total Base	151	155	146	146	145	150
Cl	100	103	101	103	101	103
HCO_3	25	28	24[22]	27	24[22]	26
PO_4 + SO_4 + Organic Acids	6**	8	7[22]	7[22]	7[22]	5
Total Acid Minus Proteinate	131	139	132	137	132	134
Total Base	151	155	146	146	145	150
−Total Acid	−131	−139	−132	−137	−132	−134
Protein Base	20	16	14	9	13	16

*Values in mEq./L. unless stated differently.

**To original 2.2 mEq./L. of PO_4 + 2.2 mEq./L. of lactate added 1.6 mEq./L. of SO_4 + traces of organic acids.

CALCULATION OF BASE BOUND BY AVERAGE NORMAL SERUM PROTEINS

Total protein	7.2 gm./100 ml.
Albumin	5.2 gm./100 ml.
Globulin	2.0 gm./100 ml.

PROTEIN BASE CALCULATED BY

1. Van Slyke *et al.* formula (1928): 18.0 mEq./L.
2. Hastings *et al.* formula (1927): 16.0 mEq./L.
3. Van Slyke *et al.* formula (1923): 14.0 mEq./L.
4. Darrow-Hartmann formula (1928): 15.5 mEq./L.

agree with formula 2 or 4 and not formula 1, regarded as the most accurate. If one accepts Sackner and Sunderman's[14] normal serum total base value of 145 mEq. per L., it would appear that the Van Slyke *et al.* formula 3[23] gives best agreement. High estimates of the protein base-binding capacity, third largest anion value, may thus account for most acid-base discrepancies. On the other hand, organic acids, mainly lactic acid, normally amount to only 1 to 2 mEq., and a divergence of this magnitude appears negligible.

SERUM TOTAL BASE IN DISEASE

For a long time, total base determinations were merely a research tool for investigations of acid-base equilibrium and were not systematically performed in clinical chemistry, such as determinations of individual electrolytes, e.g., chlorides, bicarbonates, and calcium. By the conductometric method of Sunderman[18, 19], a simple means of performing total base determinations, as well as estimations of sodium, has been provided, and a number of investigations have been carried out more recently[9, 14, 22, 25]. A survey of some data on serum total base in disease from the literature [1, 6, 11, 12, 14, 17, 20, 21] is given graphically in Figure 3.

Since various authors used different methods and different normals for serum total base, a comparison was possible only by expressing values found in disease as per cent of normality. It may be noted that largest deviations from the 100 per cent norm on the left side of the graph were reported by early authors and obviously were due in part to technical difficulties. This may also explain marked differences in the same

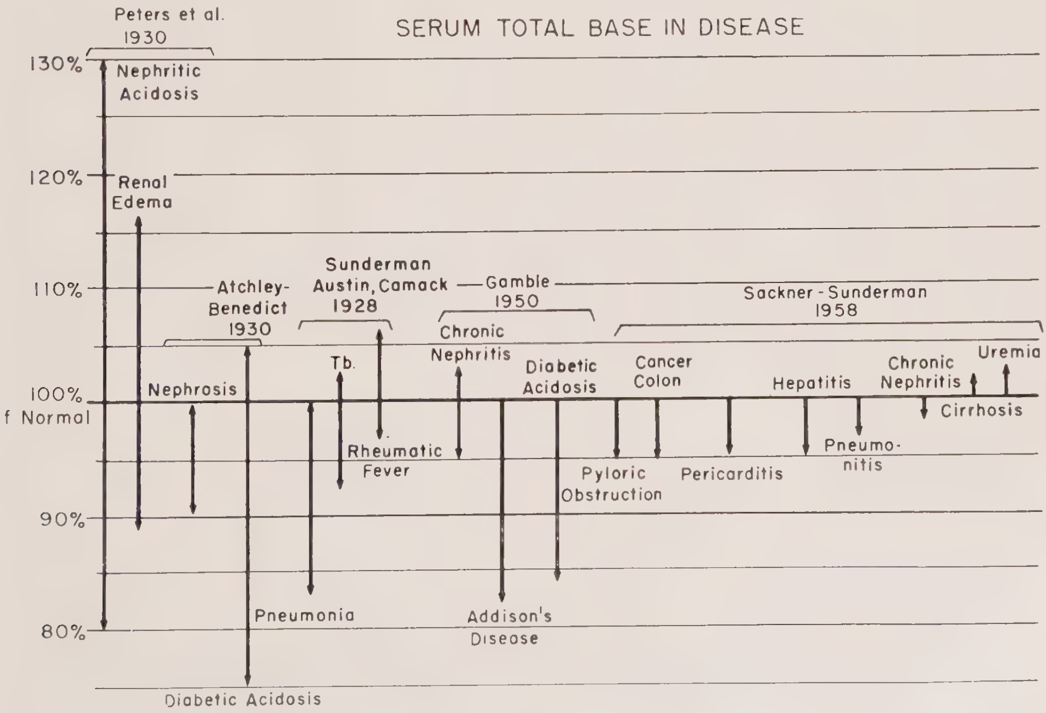

Fig. 3

condition, such as diabetic acidosis observed in 1930 and 1950, although earlier cases may have been more severe. It may further be noted that the great majority of deviations are below the norm and less often above, indicating that lowering of electrolyte concentration in the plasma compartment, either by loss or by dilution, is a more common reaction than the opposite. If deviations from the norm frequently do not appear impressive, it may be well to realize that a percentile change of serum total base reflects the same change of serum total acid and serum total electrolytes.

With only limited material available, as shown in Figure 3, not more than a few cautious conclusions are possible:

1. The most severe lowering of serum total base appears to occur in Addison's disease, lobar pneumonia, diabetic acidosis, and probably renal acidosis.
2. In chronic renal disease, serum total base appears, generally, moderately lowered, but may also be slightly increased.
3. Losses of electrolytes by vomiting, such as in pyloric obstruction, will cause decrease of serum total base.
4. In infections (pneumonitis, pericarditis, tuberculosis, hepatitis) there also appears to be depression of total serum base, while the old value of rheumatic fever seems to indicate an increase rather than a decrease.

The paucity of data indicates that much work remains to be done in order to elucidate the role of serum total base in disease.

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B. The Hald Benzidine Method for the Measurement of Total Base[2-5]

PRINCIPLE

A mixture of serum, sulfuric acid, and ferric sulfate is evaporated on a steam bath and then ignited in a muffle furnace. The sulfates are extracted from the ash with water and are precipitated with benzidine. The benzidine sulfate is filtered, dissolved in hot water, and titrated with sodium hydroxide[2, 3, 4, 5].

REAGENTS

1. Sulfuric acid, 4 N solution.
2. Ferric sulfate, 12.9 gm. diluted to 1 l.
3. Benzidine solution, four grams of benzidine are ground in a mortar with 50 ml. of normal hydrochloric acid, diluted to 250 ml. with water, filtered, if necessary, and stored in a dark bottle.
4. Sodium hydroxide solution, 0.02 N.
5. Phenolphthalein indicator, 1 per cent alcoholic solution.
6. Acetone, 95 per cent.

SPECIAL APPARATUS

1. Electric muffle furnace, which can be adjusted to a temperature of 580 to 600° C., e.g., a small "Temco" 1500 watt furnace with pyrometer, variable temperature control and heating chamber of about 5″ x 6″ x 4″.

2. Evaporating dishes of "Vycor" resistant glass 70 mm. in diameter for dry ashing, preferable to porcelain dishes which lose their glaze on repeated use.

3. Acid-washed and hardened filter paper Whatman No. 50, 9 cm., for filtering benzidine sulfate precipitate, preferable to fritted glass filters which must be assorted and tested according to pore width in order not to lose small particles of precipitate.

PROCEDURE

1. *Heating and Dry Ashing.* One ml. of serum is mixed with 1 ml. of sulfuric acid (Reagent 1) and a drop of ferric sulfate (Reagent 2) in an evaporating dish (Special Apparatus 2). The mixture is heated on a steam bath for two hours or longer to a black syrupy consistency. The evaporating dish is then placed in the electric muffle furnace (Special Apparatus 1), the temperature of which is slowly raised to 600° C. and kept at this temperature for about three hours, until white, fluffy ash is obtained and excess sulfuric acid driven off. A few black or brown spots are due to iron oxide.

2. *Extraction of Ash.* Most of the ash is easily soluble on stirring with a glass rod and is extracted by six 2-ml. portions of water. The combined extracts are centrifuged, yielding a clear supernatant solution.

3. *Precipitation with Benzidine.* Ten ml. of the supernatant solution is added drop by drop from a pipet to 2 ml. of benzidine reagent (Reagent 3) in a 100-ml. beaker while agitating. A white silky precipitate of benzidine sulfate is produced, the solubility of which is decreased by addition of 4 ml. of acetone (Reagent 6). After ten minutes the precipitate is filtered quantitatively through a 9-cm. Whatman No. 50 filter paper (Special Apparatus 3) moistened with acetone, and the precipitate on the filter is washed with three 1-ml., two 5-ml. and one 2-ml. portions of acetone.

4. *Titration.* The filter with the precipitate is next transferred with the help of a glass rod to the same 100-ml. beaker as used for precipitation. About 8-ml. of water are added and heated until all acetone is evaporated, and the entire precipitate is dissolved. After addition of a drop of phenolphthalein indicator (Reagent 5), the hot fluid is titrated with 0.02 N sodium hydroxide (Reagent 4) until a faint pink tinge persists after boiling.

5. *Calculation.* Milliequivalents of base per l. = $\frac{(\text{A-B} \times 0.02 \times 1000}{\text{V}}$, where A and B are the ml. of sodium hydroxide used for titration of the sample and a blank respectively. The latter, using water instead of serum, is carried through the entire procedure, including filtration through an identical Whatman filter paper. V is the number of ml. of serum sample. The usual corrections are made for aliquot and titration factor.

DISCUSSION

Compared to older methods, the Hald procedure incorporates several conspicuous improvements. For instance, the former sulfuric and nitric acid wet-ashing procedure is replaced by dry-ashing, avoiding time-consuming supervision, and interfering phosphorus formerly eliminated by laborious precipitating and filtering after ashing is bound as insoluble ferric phosphate by simple addition of a drop of ferric sulfate. Titration of the entire benzidine sulfate precipitate with the filter in the titration fluid, in lieu of measuring aliquots and elimination of platinum dishes, are further advantages.

SOURCES OF ERROR

Because of unequal distribution of cations between cells and serum, hemolysis must be avoided, as well as prolonged contact of serum and cells giving rise not only to chloride shift but also to diffusion of potassium from cells into serum and conversely of sodium from serum into cells.

Claims have been made that, during ashing, there were losses of cations, e.g., of magnesium according to Wright and Allison[9] and of sodium which, according to Consolazio and Dill[1], was partly "entrapped" in the iron phosphate precipitate. After painstaking investigations, Hald[4, 5] concluded that no such losses occurred but found that during ashing of whole blood, not of serum, iron phosphate caused a loss of potassium preventable by moistening the ash with hydrochloric acid, followed by evaporation on the steam bath.

The question may be raised as to complete solubility of the almost insoluble calcium sulfate. Since 0.209 gram of calcium sulfate is soluble in 100 ml. of water at 30° C.[6], a 10-ml. volume after ash extraction would dissolve up to 21 mg., while 1 ml. of serum, even in hypercalcemia, contains less than 1 mg. in terms of calcium sulfate. However, Wright and Allison[9] pointed out that the rate of solubility was slow and several minutes of stirring were necessary for solution. Further on, a problem of incomplete solubility of calcium sulfate would be encountered if the method were applied to urine or feces[7]. Another point of solubility concerns the benzidine sulfate precipitate, which must not be allowed to dry and thereby become partly insoluble.

NORMAL VALUES

Serum total base in normal adults averages 145 mEq. per L. ± 2.2 S.D. with a range of 142.0 to 149.7 mEq. per L.[8].

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The Measurement of Serum Conductivity for the Estimation of Total Base and Sodium

A. General Considerations

F. WILLIAM SUNDERMAN, M.D., Ph.D.

The estimation of the electrolyte concentration in serum by means of conductivity measurements is one of the simplest and most dependable methods available to the clinical laboratory[1, 2, 3, 4, 5, 6]. The method yields reproducible results even in the most inexperienced hands, and the measurement requires less than 1 ml. of serum. Moreover, after the measurement is made, the serum may then be used for other types of analyses. Under normal working conditions, the time required for making the measurement in serum is approximately three minutes.

If the chemical pattern of normal serum is examined (Figure 1, Chapter 1, page 4), it can be seen that 90 per cent of the total electrolyte is composed of sodium salts of which more than 90 per cent consists of chloride and bicarbonate. As shown in the figure, the bases or cations are Na, K, Ca, and Mg; and the acids or anions are HCO_3 Cl, proteins, PO_4, SO_4, and organic acids. The concentrations of the individual components are given at the sides of the diagram. The sum of the acids must equal the sum of the bases. Under normal conditions, the mean concentration of the electrolytes in serum is 146 mEq. ± 3 mEq. per L. Particular attention is called to the value of the summation of K, Ca, and Mg, which is from 10 to 11 mEq. per L., or approximately seven per cent of the total base. This summation value is maintained relatively constant during health and disease. Owing to the relative stability of this value, it is possible to obtain a rapid estimation of the sodium concentration of serum, adequate for clinical purposes, by merely subtracting 10.9 mEq. from the total base values. Since sodium constitutes 93 per cent of the total base, any significant changes in concentration of sodium are reflected in the total base values.

Although flame photometry offers a potentially simple and rapid method for estimating sodium, nevertheless, it is important to recognize that this procedure is far from fool-proof and that technical difficulties, peculiar to each

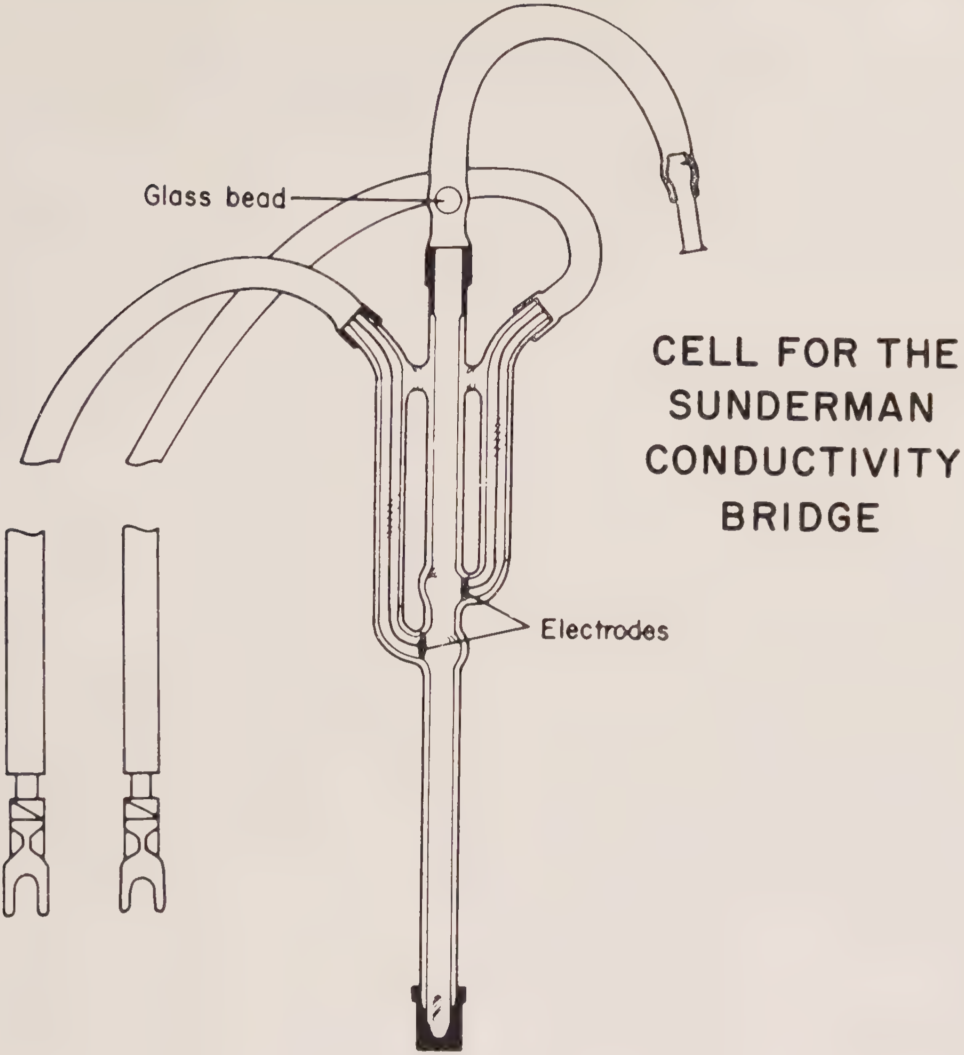

Fig. 1

instrument, must be evaluated critically. Because of the importance of determining not only sodium but also the concentration of total electrolytes in the serum, conductivity measurements are particularly useful for estimating both total base and sodium.

The concept of employing conductivity measurements for estimating the serum electrolytes goes back a number of years. As early as 1898, Bugarszky and Tangl[7] attempted to employ conductivity measurements for this purpose. These workers found that the conductivity values of serum were lower than anticipated from the concentrations of salt that were present, and they showed that the diminution was due to the presence of serum protein, for which they tried to make a correction.

Conductivity measurements, however, did not reach any degree of importance until 1922, when Christiansen[8] introduced an instrument which he called an *ionometer*. The Christiansen ionometer is a 110-volt, direct current, spring voltmeter connected in series with a capillary type of conductivity cell into which is introduced a few drops of serum. Since direct current is used, it is necessary to prevent polarization effects (that is, electrolysis at the electrodes). Such effects are minimized by using capillary types of cells that give high cell constants around 200. Christiansen calibrated the scale of his ionometer with NaCl solutions of known concentration.

In order to reduce polarization effects and to discontinue the use of direct current while retaining the simplicity of operation and convenience of the ionometer, a Leeds and Northrup sugar-ash bridge was modified[1] for conductivity measurements on serum. The sugar-ash bridge is an instrument used for measuring the presence of impurities in sugar solutions. The bridge is a 60-cycle, 110-volt, Wheatstone bridge with a galvanometer inserted as a null-point indicator.

The design of the bridge and cell have undergone modifications[5]. The bridge now in use* operates on a 110-volt, 60-cycle current and is driven by a 1000 cycle electronic tube oscillator. A cathode ray tube (magic eye) serves as a null-point indicator and provides readings unaffected by vibrations. The design of the cell has also been changed. The capacity of the cell is reduced to 0.5 ml., and the electrodes are imbedded in the walls of the cell so that obstructive bubbles are eliminated (Fig. 1).

The first study of the correlation of total base in serum with conductivity was made in 1926, by Sunderman, Austin, and Camac[9]. Although the correlation was linear, there was considerable scattering of the values. It became apparent that if conductivity measurements were to become meaningful in clinical medicine, the correlation of conductivity and total electrolytes needed detailed study. At first, a study[9] was made of the conductivity properties of synthetic mixtures of electrolytes and lyophilized serum proteins from which the salts had been removed previously by dialysis. Subsequently, an investigation was undertaken of the correlation between chemically determined total base and the measured values for serum conductivity and serum protein[2].

As previously mentioned, Bugarszky and Tangl[7] found that the observed conductivity of serum was less than anticipated owing to the presence of serum protein. In studying this effect, Palmer, Atchley, and Loeb[10] showed that the conductivity of a solution of NaCl was decreased a uniform amount for each increment of protein (egg albumin) that was added.

On the other hand, Gram and Cullen, in 1923[11] found that the conductivity in serum was not decreased by a uniform amount by the presence of protein. When their data were analyzed, the corrected conductivity values could be best fitted by use of the following type of equation:

Equation I**

$$C_c = \frac{100\,C_o}{100 - k\,Pr}$$

This equation is based on the assumption that the observed conductivity is decreased percentilely by each increment of protein. An average value of 2.2 was found by Gram and Cullen to be the constant for this equation. When the Gram and Cullen formula was applied to total base measurements, the calculated total base values gave a mean percentile deviation of ± 3.3 per cent from the analyzed values. A percentile deviation of ± 3.3 per cent is obviously not acceptable for clinical purposes.

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*Conductivity Bridge, Sunderman, #4031 B, A. H. Thomas Company, Vine Street at Third, Philadelphia, Pa., 19107.

**See pg. 169 for identification of abbreviations.

Our studies finally led us to consider the application of the Onsager equation for correcting conductivity values[2]. It is known that the mobility of an ion is decreased when the ionic concentration is increased. An equation for this effect was derived by the Scandinavian physical chemist, Onsager[12]. The constants in the Onsager equation involve the effects of viscosity, dielectric constant, temperature and ionic concentration. By making the simple assumption that the viscosity is a function of the protein concentration, an empirically simplified form of the Onsager equation was derived as follows:

Equation II

$$C_c = C_o + \frac{\overline{\mu \sqrt{\mu k}}}{Pr} + \overline{\mu \sqrt{\mu k_1}}$$

C_c = corrected conductivity (mhos x 10^3 at 25° C).
C_c = corrected conductivity (mhos x 10^3 at 25° C).
K, k_1 = constants.
Pr = serum protein (gm./100ml.).
μ = ionic strength.

Replacing determined constants in

Equation II, then

Equation III becomes

Total base

mEq./L.

$$= 10.19\, C_o - \frac{69.45}{Pr} + 35.16$$

The form of this equation is in accord with the observations of diminishing conductivity with increasing concentrations of protein. Using this equation, the observed values in a series of forty-seven samples of serum gave a mean percentile deviation of ± 1.5 per cent.

The protein values in Equation III were computed by means of the nitrogen factor: 6.25. Within the past few years, it has been shown that the protein nitrogen, multiplied by a factor of 6.54, yields more accurate values for estimating total protein in serum[13]. Therefore, Equation III has been modified to the following:

Equation IV

Total base

mEq./L.

$$= 10.19\, C_o - \frac{72.67}{Pr} + 35.16$$

Equation IV is the equation which is now used for our calculations of total base. Although any reliable method may be used to obtain the value for the serum proteins (Pr) in this equation, the refractometric method is recommended for rapidity and convenience[14].

When total base estimations are used as a rapid guide for replacement therapy, the measurement of serum protein is not always necessary. Under such conditions, calculation can be made on the assumption of an average normal value for serum protein. If the serum protein concentrations are within the range of values for most hospitalized patients an error of less than 1 per cent is introduced when a mean value of 6.7 grams per 100 ml. is assumed. If the concentration of serum protein is decreased to 5.2 grams per 100 ml., an error of only 2 per cent is introduced when a mean value is assumed. For many clinical purposes, therefore, the

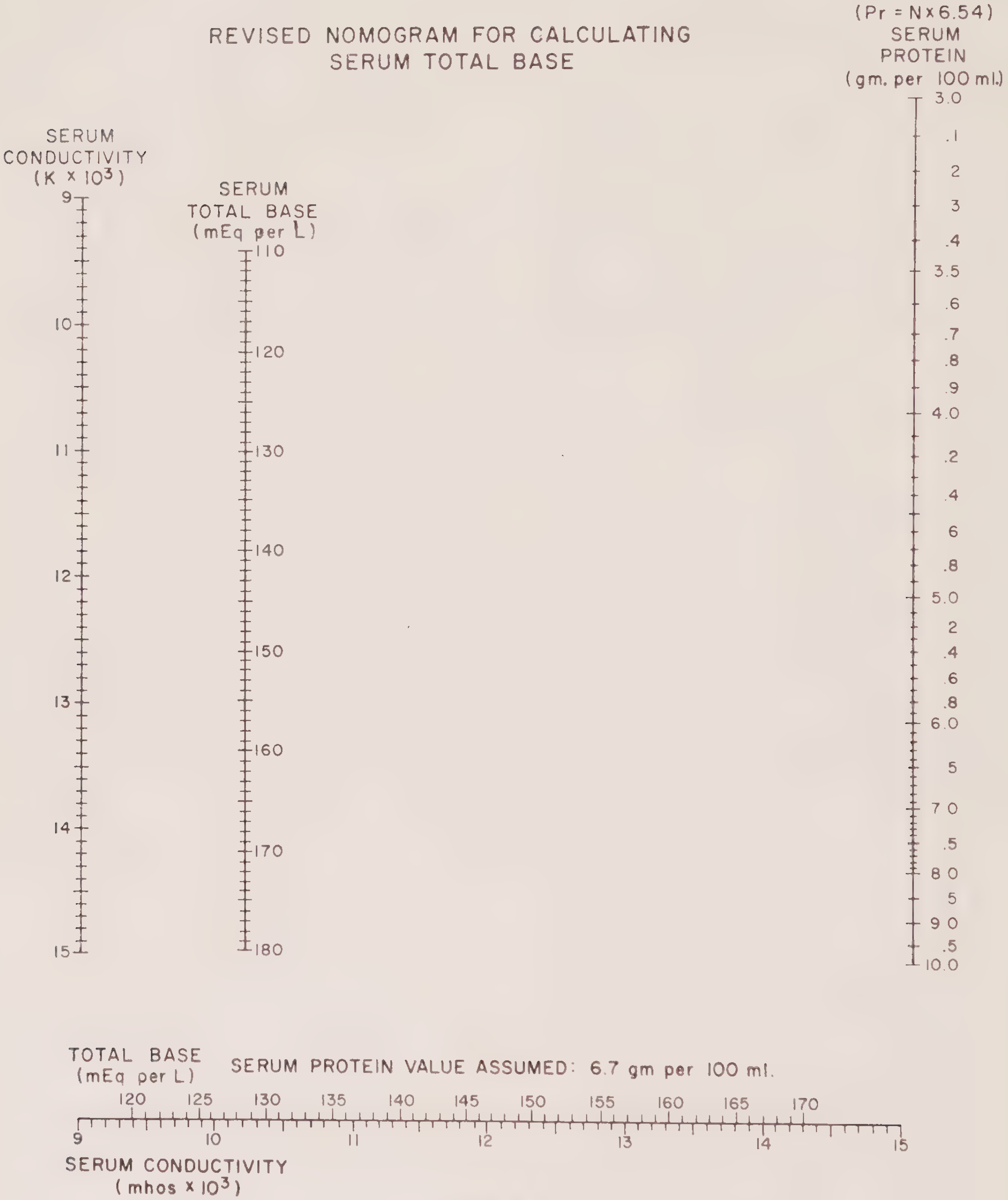
REVISED NOMOGRAM FOR CALCULATING SERUM TOTAL BASE
SERUM CONDUCTIVITY (K × 10³)
9
10
11
12
13
14
15
SERUM TOTAL BASE (mEq per L)
110
120
130
140
150
160
170
180
(Pr = N × 6.54) SERUM PROTEIN (gm. per 100 ml.)
3.0
.1
2
3
.4
3.5
.6
.7
.8
.9
4.0
.2
.4
6
.8
5.0
2
.4
.6
.8
6.0
5
7 0
.5
8 0
5
9 0
.5
10.0
TOTAL BASE (mEq per L)
SERUM PROTEIN VALUE ASSUMED: 6.7 gm per 100 ml.
120
125
130
135
140
145
150
155
160
165
170
9
10
11
12
13
14
15
SERUM CONDUCTIVITY (mhos × 10³)

Fig. 2

total base may be calculated with sufficient accuracy without making the additional measurements of protein.

For the calculation of total base without the protein correction, the following equation may be used:

Equation V

$$\text{Total base}_{\text{mEq. per L.}} = 10.19\ C_o + 24.3$$

A table of conductivity and protein values has been prepared to facilitate the estimation of total base in everyday laboratory work (Table I). For convenience, a nomogram is also included (Fig. 2).

A number of serums studied by Sackner and Sunderman[6] contained severe dislocations of the protein components. In these serums, the total base values determined by conductivity measurements were all in good agreement with those obtained by chemical analyses. In addition, the presence of abnormal concentrations of non-electrolytes, such as urea, glucose, cholesterol, and bilirubin did not introduce significant errors. For clinical purposes, the conductivity method for estimating the total electrolytes in serum is rapid, dependable and economical of materials.

TABLE I TOTAL BASE IN SERUM (mEq. PER L.)

SERUM CONDUCTIVITY	SERUM PROTEIN (GRAMS PER 100 ML.)											
($K \times 10^3$)	*4.0*	*4.5*	*5.0*	*5.5*	*6.0*	*6.5*	*7.0*	*7.5*	*8.0*	*8.5*	*9.0*	*9.5*
10.0	118.9	120.9	122.5	123.9	125.0	125.9	126.7	127.4	128.0	128.5	129.0	129.4
10.2	120.9	122.9	124.5	125.9	127.0	127.9	128.7	129.4	130.0	130.5	131.0	131.4
10.4	123.0	125.0	126.6	128.0	129.1	130.0	130.8	131.5	132.1	132.6	133.1	133.5
10.6	125.0	127.0	128.6	130.0	131.1	132.0	132.8	133.5	134.1	134.6	135.1	135.5
10.8	127.1	129.1	130.7	132.1	133.2	134.1	134.9	135.6	136.2	136.7	137.2	137.6
11.0	129.1	131.1	132.7	134.1	135.2	136.1	136.9	137.6	138.2	138.7	139.2	139.6
11.2	131.1	133.1	134.7	136.1	137.2	138.1	138.9	139.6	140.2	140.7	141.2	141.6
11.4	133.2	135.2	136.8	138.2	139.3	140.2	141.0	141.7	142.3	142.8	143.3	143.7
11.6	135.2	137.2	138.8	140.2	141.3	142.2	143.0	143.7	144.3	144.8	145.3	145.7
11.8	137.2	139.2	140.8	142.2	143.3	144.2	145.0	145.7	146.3	146.8	147.3	147.7
12.0	140.3	141.3	142.9	144.3	145.4	146.3	147.1	147.8	148.3	148.9	149.4	149.8
12.2	141.3	143.3	144.9	146.3	147.4	148.3	149.1	149.8	150.4	150.9	151.4	151.8
12.4	143.4	145.4	147.0	148.4	149.5	150.4	151.2	151.9	152.5	153.0	153.5	153.9
12.6	145.4	147.4	149.0	150.4	151.5	152.4	153.2	153.9	154.5	155.0	155.5	155.9
12.8	147.4	149.4	151.0	152.4	153.5	154.4	155.2	155.9	156.5	157.0	157.5	157.9
13.0	149.5	151.5	153.1	154.5	155.6	156.5	157.3	158.0	158.6	159.1	159.6	160.0
13.2	151.5	153.5	155.1	156.5	157.6	158.5	159.3	160.0	160.6	161.1	161.6	162.0
13.4	153.5	155.5	157.1	158.5	159.6	160.5	161.3	162.0	162.6	163.1	163.6	164.0
13.6	155.6	157.6	159.2	160.6	161.7	162.6	163.4	164.1	164.7	165.2	165.7	166.1
13.8	157.6	159.6	161.2	162.6	163.7	164.6	165.4	166.1	166.7	167.2	167.7	168.1

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B. Estimation of Serum Total Base by the Sunderman Conductivity Method

GEORGE F. STEVENSON, M.D.

PRINCIPLE

Specific conductance of a liquid is defined as the conductance between two 1 cm. square electrodes placed 1 cm. apart. The specific conductance of serum varies directly with its total base content, if the protein content of the serum is constant. When the protein content is significantly decreased, a correction theoretically must be made in specific conductance values; in actual use this is rarely necessary. The specific conductance of serum may be measured accurately, rapidly and economically using the Sunderman conductivity apparatus, the total base and sodium values obtained by this method being remarkably reproducible.

REAGENTS

1. *Sodium Chloride, 140 mEq./L. Aqueous Solution*: Fused sodium chloride (8.183 gm.) is dissolved in distilled water and diluted to 1 liter. This solution has a specific conductance of 14.58 x 10^{-3} mhos at 25° C.

2. *Sodium Chloride, 0.85 Per cent Aqueous Solution.*

3. *Hydrochloric acid, 10 per cent v/v.*

4. *Electrode "Decoating" Solution*

(Aqua Regia): Three parts of concentrated hydrochloric acid are mixed with one part of concentrated nitric acid.

5. *Electrode Replatinizing Solution*: 3 per cent platinic chloride containing 0.025 per cent neutral lead acetate.

SPECIAL APPARATUS*

1. *Conductivity Bridge*: The Sunderman conductivity bridge is a modified Wheatstone bridge in which the unbalanced voltage is amplified and fed to a cathode ray ("magic eye") null indicator. After the temperature and conductivity cell constant compensators have been adjusted, the bridge is balanced, using a conductance dial calibrated in reciprocal ohms (mhos). The result is read directly from the dial with a scale range of 5 to 21 mhos x 10^{-3}.

2. *Conductivity cell*: The Sunderman conductivity cell is a specially constructed pipet having a wide bulb which contains two platinum electrodes that are attached to leads for connection to the terminals of the conductivity bridge. These cells have constants in the neighborhood of 10. When not in use, the cell should be kept filled with CO_2-free distilled water.

3. *Water Bath, 25° C. and Thermometer* graduated in 0.1° between 20 and 30°.

STANDARDIZATION AND CARE OF APPARATUS

1. *Conductivity Cell Constant*: To determine the cell constant, the conductivity pipet is filled with the 140 mEq. per L. sodium chloride solution at a constant temperature of 25°. The conductance dial is set at 14.58. The null indicator is then balanced, using the cell compensator control. When balance is obtained the cell constant is read directly from this dial.

2. *Cleaning and replatinization of conductivity cell electrodes*: When the null indicator or "magic eye" shadow becomes hazy at balance, the conductivity cell requires cleaning. To clean the pipet it is filled with the 10 per cent hydrochloric acid solution and allowed to stand for three minutes, followed by repeated rinsing with distilled water. If this cleaning procedure does not correct the appearance of the null indicator, the electrodes must be replatinized. Replatinization is also needed when the electrodes become polarized as indicated by instability of the null indicator.

To replatinize the electrodes, the pipet is first filled with aqua regia to remove the original coating. It is then rinsed well with distilled water. Next the conductivity pipet is filled with the replatinizing solution and the terminals of the pipet are connected to three 1.5-volt dry cell batteries assembled in series. This electrolysis is continued for ten to fifteen minutes or until an even black velvety coat of platinum is deposited on each electrode. During this procedure the direction of the current should be reversed every thirty seconds; this may be accomplished easily using a double pull-double throw electric switch. After replatinization the electrodes should stand in water for two to three days before use, the water being changed four to five times during this period.

3. *Conductivity Bridge Standardization*: Two resistors, 820 ohms ± 5 per cent and 82 ohms ± 1 per cent are connected across terminals 1 and 2,

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*Apparatus and reagents are obtainable from Arthur H. Thomas Company, Philadelphia, Pennsylvania.

respectively. The cell compensator control is adjusted to 8.9, and the temperature control is set at twenty-five. When the conductance dial is then balanced it should read ten.

PROCEDURE

1. The conductivity bridge is turned on and the conductivity cell is attached to terminals one and two. The instrument should be permitted to warm up for at least five minutes.
2. The conductivity cell is filled with serum so that both electrodes are covered completely. In filling this pipet, the rubber tubing is pulled away from the glass bead valve while drawing in the serum.
3. The rubber cap is placed over the tip of the pipet, and the cell is placed in a water bath at 23° to 27° C. so that the bulb containing the electrodes is immersed completely.
4. The temperature of the water bath is determined to within 0.1° C.
5. The temperature compensator control is adjusted to this temperature value.
6. The cell compensator control is adjusted to the cell constant value.
7. The instrument is then balanced by means of the conductance control; the balance point is obtained when the dark portion of the "magic eye" indicator reaches its greatest width.
8. When balance is obtained, the specific conductance value is indicated on the conductance dial (mhos x 10^{-3}).
9. The serum is expelled, and the pipet is rinsed at once, first with 0.85 per cent sodium chloride solution, and then with distilled water. When not in use, the pipet should be kept filled with CO_2-free distilled water. Failure to rinse the pipet first with physiologic saline will permit precipitation of protein on the electrodes.

CALCULATIONS

Total base may be calculated either from the nomogram (page 168) or the table (page 171): the concentration of sodium may be obtained by subtracting 10.9 mEq. per L. from the total base values.

DISCUSSION

The specific conductance method of measuring serum sodium is one of the most accurate and most reliable methods available to the general laboratory for routine use. It has failed to receive wide acceptance because there is no equally simple, accurate and rapid method for estimating serum potassium. As a result, the method has been used principally for checking serum sodium results obtained by other methods and in standardization of sodium procedures.

The estimation of total base by this method is precise; values differ from those obtained by the chemical method by an average of only 1.5 per cent. Reproducibility of results obtained in routine laboratory use with well standardized equipment probably does not exceed 1 per cent.

NORMAL VALUES

The normal range of serum total base is 142 to 149 mEq./L., with a mean of 146 mEq./L., in the resting, fasting adult. Serum sodium determined by this method has the same range of normal values as serum sodium by either flame photometry or by the ashing procedure (mean 135 mEq./L.).

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The Measurement of Osmolality of Serum

A. The Freezing Point Method for the Measurement of Osmolality [1]

F. WILLIAM SUNDERMAN, M.D., Ph.D.

INTRODUCTION

The method most frequently employed for the estimation of osmotic pressure in biological fluids is the cryoscopic or freezing point method. In an aqueous solution, the freezing point is the equilibrium temperature between ice and the solution. Several different methods may be utilized to measure the freezing point:

1. In the Beckman method[2, 3], an equilibrium vessel is immersed in an air bath at a temperature two or three degrees below the freezing point. The aqueous solution in the equilibrium vessel is thereby supercooled. A part of the solvent (water) is frozen out. This may be initiated by seeding or stirring. The supercooling and the increased concentration of solutes owing to the freezing out of solvent (water) tend to give unduly high depressions. Various attempts have been made to compensate for the effect of supercooling. These compensations are usually made by correcting the observed freezing points by comparisons with known NaCl solutions[4]. In general, methods employing the principle of supercooling are not recommended for measurements in biological fluids.

2. For precise measurements, an aqueous solution of a solute may be mixed with small pieces of ice in an equilibrium vessel and the solution stirred efficiently. The equilibrium vessel is placed in a water bath at 0°. When equilibrium is reached, a sample of the solution (without ice) is quickly taken from the equilibrium vessel, and the precise concentration of the solute in the solution is determined by analysis. Some ice is melted in bringing the solution and ice to the equilibrium temperature; hence, this method cannot be used to determine the freezing point of a solution such as serum. However, by using this method, a curve may be plotted of concentrations of solute against their respective freezing point depressions[5, 6].

3. A number of methods have been devised in which the sample is first

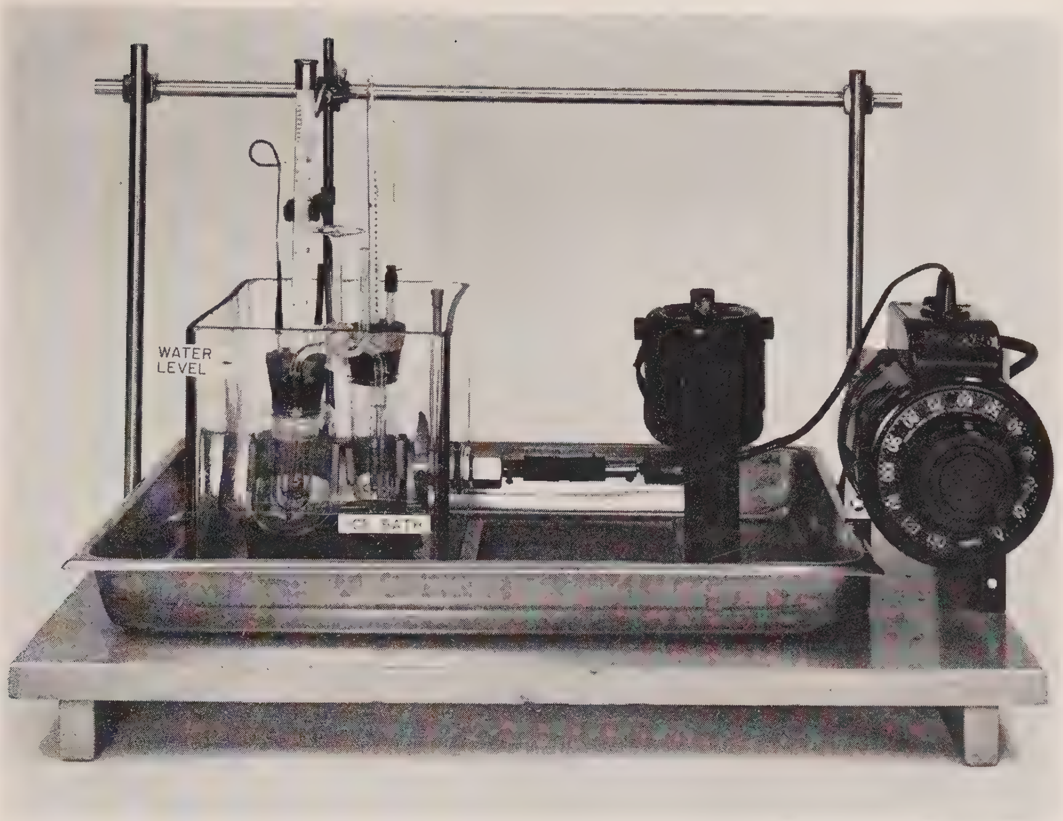

Fig. 1. Stadie-Sunderman Freezing Point Apparatus in Ice Bath with Motor-driven Stirrer

frozen and then slowly warmed until the last crystal of ice has disappeared. The temperature at which this disappearance occurs is taken as the freezing point.[7, 8, 9, 10, 11] The main difficulties with methods of this type involve the temperature control and the timing of the disappearance of the ice.

4. The Stadie-Sunderman Method[1] differs in principle from the three types of measurements that have been described. The method avoids errors owing to supercooling, concentration of solutes, and the timing of disappearance of ice crystals. The method is well-adapted for the determination of the freezing point in biological fluids.

PRINCIPLE OF THE STADIE-SUNDERMAN METHOD

The biological fluid is cooled to a temperature within 0.1 to 0.2° of the freezing point. In the case of serum, the range of the freezing point is between −0.49 and −0.69°; therefore, the serum is cooled to approximately −0.45°. After this temperature is reached, the serum is transferred to a precooled equilibrium vessel containing several pea-sized pieces of ice, a Beckman thermometer, and a stirrer. The precooling of the serum and the equilibrium vessel with its contents minimizes the melting of ice necessary to attain equilibrium. After a series of transfers, thermal equilibrium

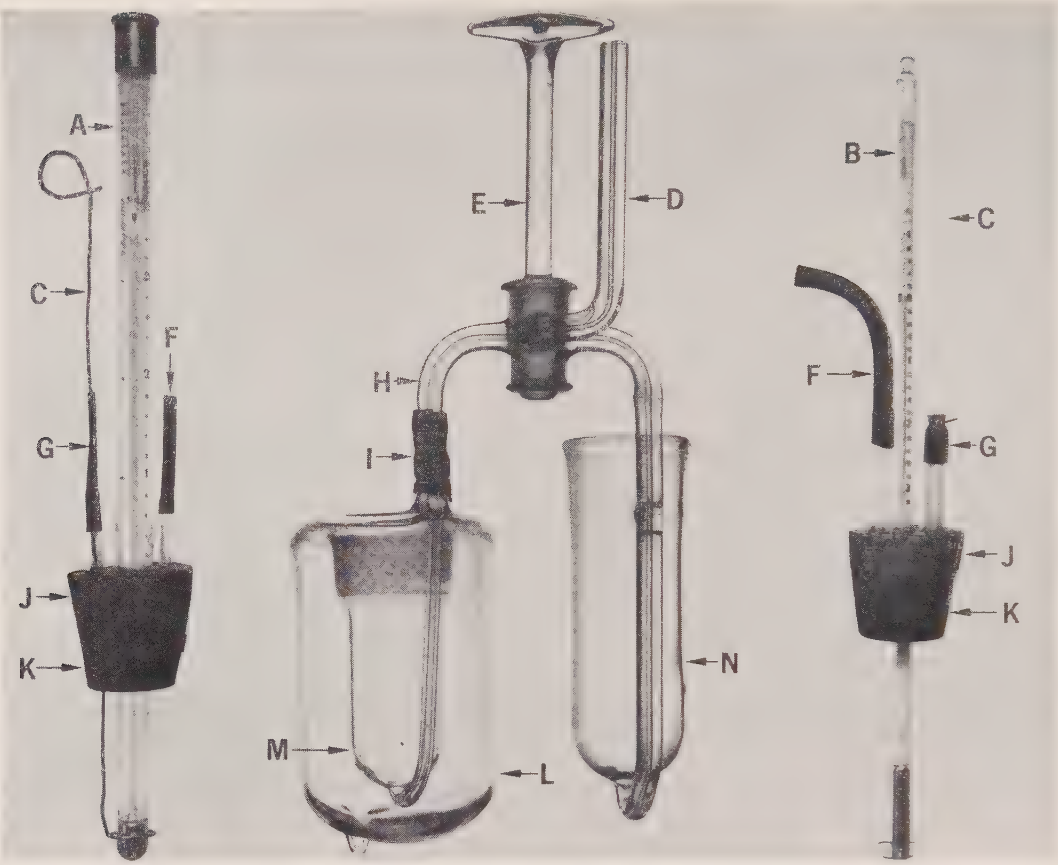

Fig. 2. Details of Stadie-Sunderman Freezing Point Apparatus.

	A — Beckman Thermometer, ±0.001°		H — Connecting Tube
	B — Beckman Thermometer, ±0.01°		I — Rubber Tube Coupling
(2)	C — Stirrer	(2)	J — Sealing Wax Support
	D — Suction Tube	(2)	K — Rubber Stopper
	E — 3-Way Stopcock		L — Vacuum Jacket
(2)	F — Air Inlet		M — Equilibrium Vessel
(2)	G — Rubber Sleeve		N — Cooling Vessel

between ice and serum is attained. The temperature of this state of equilibrium represents the freezing point.

APPARATUS

The details of the apparatus are shown in Figures 1 and 2.

The "equilibrium vessel" consists of a tube 15 x 30 mm. surrounded by a vacuum jacket. A rubber stopper holds a Bureau-of-Standards, certified Beckman thermometer reading to ±0.001°, a glass tube to guide the stirrer, and a second smaller glass tube to act as an air inlet. When the stirrer is in place, a small piece of rubber tubing fits snugly over it and its holder, acting as a rubber sleeve to prevent condensed moisture from the air from running into the equilibrium vessel. The thermometer, stirrer and inlet tube, are rigidly supported in the rubber stopper by an extension of sealing wax, which is made by pouring melted sealing wax into a paper cylinder mold around the stopper. A sleeve of rubber tubing around the top of the equilibrium vessel forms the outside support for a mercury seal. This seal and the rigid setting of the thermometer prevent leakage from the brine bath in which the entire apparatus is immersed.

The bottom of the equilibrium vessel is fused to a 2-mm. glass tube which passes up and through the jacket. Rubber tubing is used to connect the equilibrium vessel through a three-way stopcock to the "cooling vessel." The stopper for the cooling vessel is fitted with a stirrer and air inlet as well as a small Beckman-type thermometer reading to ±0.01°. The two main units of assembled apparatus are held in place by two strong brass spring clamps supported by a ⅜ inch upright brass rod, which also carries a bakelite base for inferior support. This assembly can be raised and lowered at will into a glass container 7 x 8 x 7 inches. The glass container is pierced at one end for the shaft of a motor-driven stirrer, the bearing of which is housed in a small stuffing box. The container is filled with finely-chopped ice and water and vigorously stirred to attain a temperature of 0°. Saturated brine may be added to the ice bath to obtain temperatures below zero.

PROCEDURE

1. Ice is crushed and washed with distilled water. Ten to fifteen pea-sized pieces of ice are selected and placed in the vacuum-jacketed equilibrium vessel with 6 to 8 ml. of distilled water sufficient to cover the bulb of the Beckman thermometer.

2. Serum is placed in the cooling vessel, and the equilibrium and cooling vessels are connected and placed in the apparatus stand.

3. The apparatus is then placed in a bath which is filled with a mixture of ice and water, and the mixture is stirred. After thirty to forty-five minutes, when the entire apparatus and contents have attained 0°, the ice-water mixture in the equilibrium vessel is stirred, and the readings of the thermometer are taken at intervals of four to five minutes until constancy of ±0.001° is attained. Three or four readings usually suffice. After the proper stem correction is made, the last reading represents the value of 0° for the thermometer that is used. The water in the equilibrium vessel is then removed by suction through the 3-way stopcock. Ice, free of water, remains in the equilibrium vessel.

4. The bath temperature is now lowered to approximately −0.45° by adding saturated brine, a few ml. at a time. The desired temperature of the bath may be maintained with ease by the occasional additions of brine. The serum in the cooling vessel is now cooled to the bath temperature.

5. The stopcock is turned to connect the equilibrium vessel to the cooling vessel. By means of gentle air pressure applied by mouth to the air-inlet tube, approximately 2 ml. of serum are transferred from the cooling vessel to the equilibrium vessel.

6. After thermal equilibrium is attained in the equilibrium vessel, the wash serum is removed by suction. Another 2 ml. of serum are then transferred from the cooling vessel to the equilibrium vessel.

7. After the second washing of the ice with serum, approximately 6 ml. of

serum are transferred into the equilibrium vessel for the final reading. The outside bath temperature is then adjusted to the approximate temperature of the equilibrium vessel.

8. After gentle stirring of the mixture of ice and serum in the equilibrium vessel, the freezing point is read. A temperature which is stable to ±0.001° for five minutes is accepted as the final reading for the freezing point. Since there is no dilution of the serum from the last transfer, the serum may be removed from the equilibrium vessel and used for other analyses.

PRECAUTIONS

Accurate freezing points are obtained only when the equilibrium vessel is immersed in a bath at approximately the freezing temperature. Even a vacuum-jacketed equilibrium vessel does not prevent loss of heat from the solution. Experience with bath temperatures reveals no difference in the freezing point when the temperature of the bath is kept to within ±0.01° of the freezing point.

Leakage of brine from the bath into the equilibrium vessel may be a source of error. Well-fitted stoppers with extensions of sealing wax to ensure rigid support will help to eliminate this source of error. Mercury seals may be used as an added safety measure against leakage. As a means of detection of dilution or contamination of the serum, conductivity measurements are made on the serum before and after the freezing point determination.

Large errors may be introduced by using alcohol and ether to dry the vessels with which the serum or biological fluid comes into contact.

STEM CORRECTION

For careful freezing-point measurements, it is essential to apply stem corrections[12]. The following equation may be used to calculate the stem correction:

$$\text{Stem Correction} = d \bullet a\,(t - t').$$

The symbol 'd' represents the degrees of the exposed portion of the mercury thread; 'a' is the coefficient of expansion of mercury in glass (average value = 0.00016); 't' is the temperature of the exposed part of the stem; and 't″ is the temperature of the solution in which the bulb is emersed.

SUMMARY

The Stadie-Sunderman method for the measurement of the freezing-point of serum has been described. The method avoids errors owing to supercooling and concentration of solutes and is well-adapted for measurements on biological fluids.

The range of normal values for the freezing-point depression is 0.535 to 0.555°.

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B. The Measurement of Serum Osmolality by Vapor Pressure Osmometry

Harry C. Ehrmantraut, Ph.D.

INTRODUCTION

The osmotic pressure of an aqueous solution, such as blood serum, can, in principle, be measured by direct or indirect methods. The direct approach would involve using a semi-permeable membrane, which would allow water to pass through, but would restrict the movement of all solids including electrolytes. In the past, this has not been practical because membranes with sufficiently small pores were not available. However, recent developments, primarily for the use in desalination of water, have led to membranes which may at some time permit the direct measurement of the total osmolality of serum.

The indirect methods involve measurement of freezing point, boiling point, or vapor pressure of the serum. Because of the presence of protein, measurement of boiling point is obviously not useful. Freezing point is a method which is most widely used, and in the hands of an experienced physical chemist, can provide extremely reliable data, as found in the *International Critical Tables*, for a variety of solutes. Unfortunately, the limitation lies in the fact that in order to establish a temperature equilibrium for measurements of freezing point depression, some solid phase (i.e., ice) must be present. In forming this ice, the concentration of the solution being determined must be altered by the amount of ice which is formed in the equilibrium slush. Physical chemists avoid this error by arriving at an equilibrium mixture of solid and liquid phase, then withdrawing a sample of the liquid phase and determining the concentration on that solution. Since this is not practical to do in clinical serum measurements, these figures may be high by a consistent amount. (See discussion in Section A.)

None of these objections apply to the determination of vapor pressure as is done in thermoelectric osmometry.

PRINCIPLE OF THERMOELECTRIC OSMOMETER

The principle of this method can best be described by visualizing an arrange-

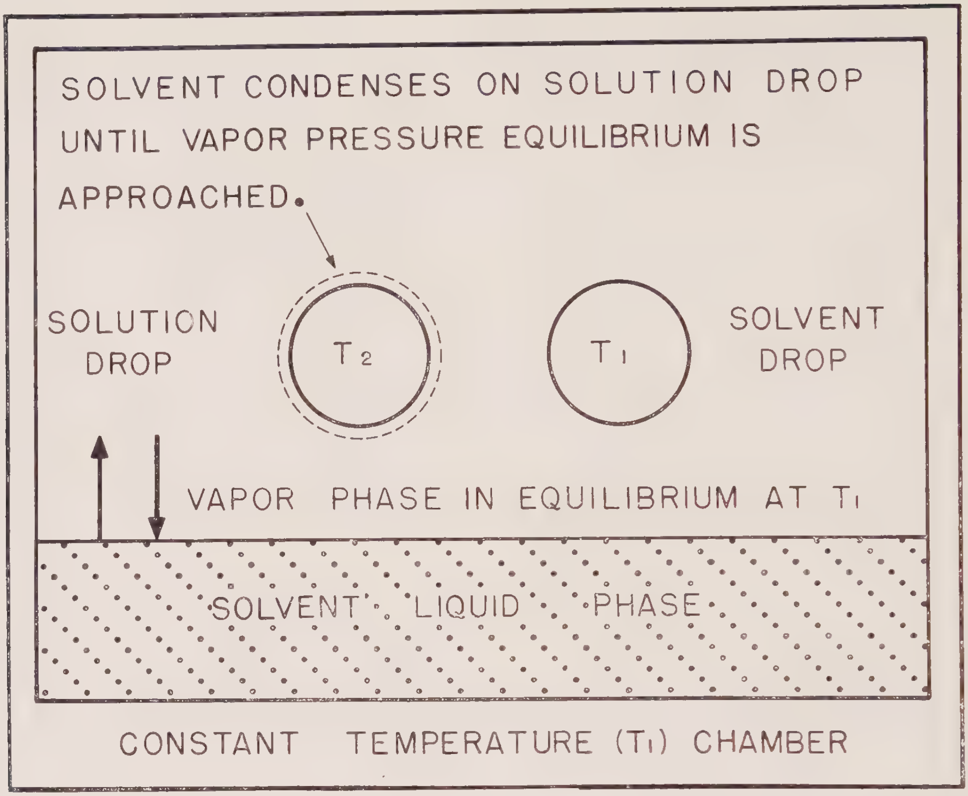

SOLVENT CONDENSES ON SOLUTION DROP UNTIL VAPOR PRESSURE EQUILIBRIUM IS APPROACHED.
SOLUTION DROP
T2
T1
SOLVENT DROP
VAPOR PHASE IN EQUILIBRIUM AT T1
SOLVENT LIQUID PHASE
CONSTANT TEMPERATURE (T1) CHAMBER

Fig. 1

ment such as is illustrated in Figure 1, which shows a relatively small chamber surrounded by a very well-thermostatted heat sink, kept at temperature T_1. If a solvent, such as water, is placed in the bottom of this chamber, it will rapidly establish vapor equilibrium with the air space of the chamber so that both the vapor and the liquid are now at T_1. If a drop of water is then introduced into the air space, this also will assume the same temperature through evaporation or condensation. If a drop of solution, i.e., water containing salts, is introduced into this chamber, it will have a lower vapor pressure than the pure water because of its dissolved solutes. Water vapor will tend to condense on the drop, but by so doing, and releasing the heat of vaporization, this condensation will raise the temperature of that drop. As the temperature is raised, the vapor pressure of the solution will also increase, and this process will proceed until the vapor pressure of the solution is in equilibrium with the vapor pressure of the surrounding water. The drop will now be at a new temperature, T_2. For an ideal situation, temperature difference between these two drops is predicted by the Clausius-Clapeyron equation which shows that

$$\Delta T = T_2 - T_1 = \frac{RT^2 M}{\lambda\, 1000}$$

where

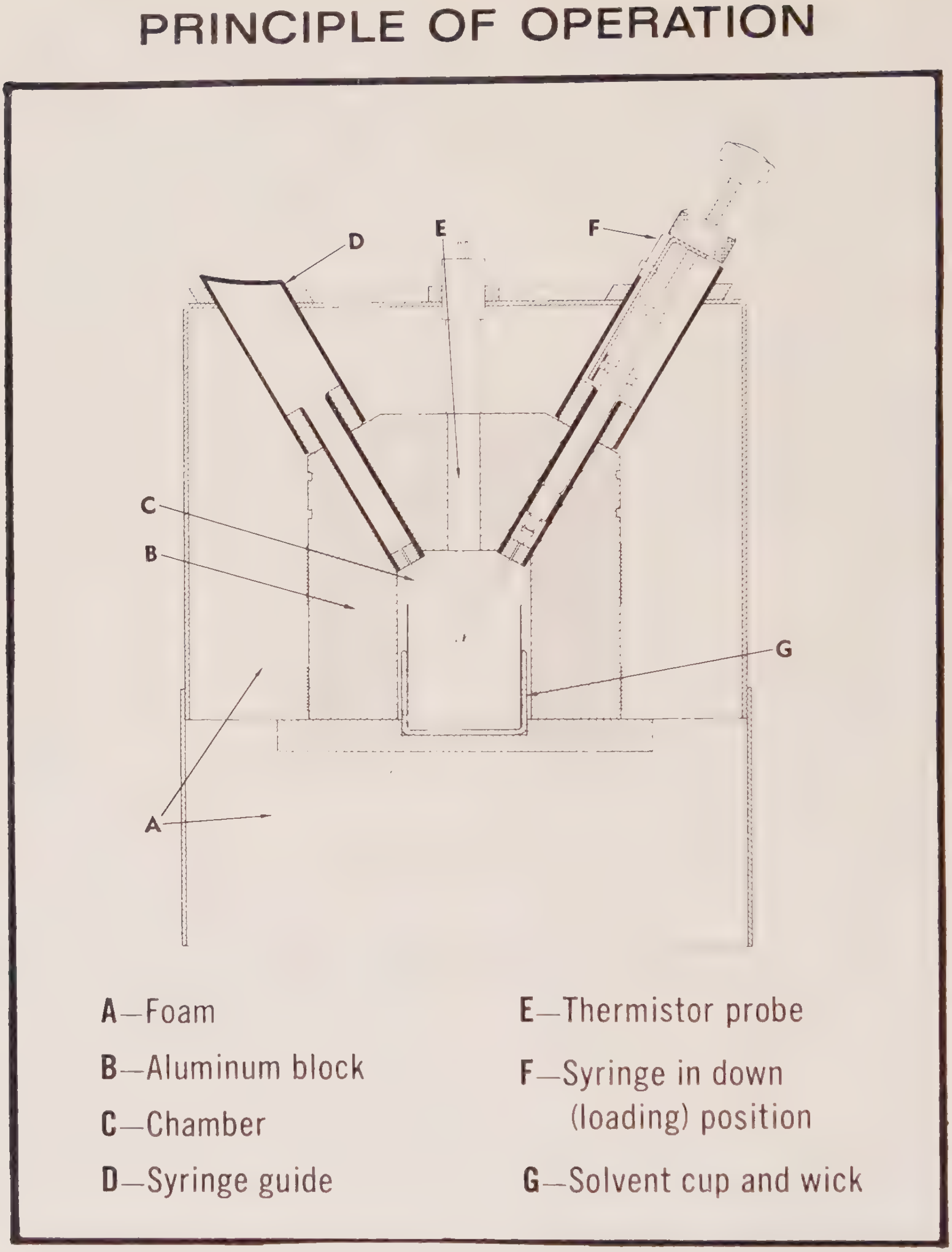
T_1 = the temperature of the chamber and solvent drop,
T_2 = the temperature of the serum or solution drop,
M = the molality of the solution,
λ = the heat of vaporization per gram of solvent,
R = gas constant,
T = absolute temperature.

For the real case, small heat losses occur causing small additional amounts of solvent to condense to give rise to a steady-state temperature differential slightly less than that predicted by the equation. The dilution effect is extremely small, being less than 0.1 per cent for typical measurements. Theoretical treatments for the efficiency of this type of osmometer have been published repeatedly[2, 9]. The efficiency is a function of the thermodynamic parameters of the solvent, temperature, and the geometry of the chamber. It is independent of the solute and only slightly dependent on the magnitude of ΔT. Thus, the activity of solutions can be determined simply by measuring the steady-state temperature differential between the unknown and the reference samples and converting it to thermodynamic parameters by means of calibration curves established with solutions of known activities. Several recent papers[3, 19] have proven the validity of this approach to the determination of activity coefficients for salts such as NaCl, Na_2SO_4, $Al_2(SO_4)_3$, KH_2PO_4, etc., and bases such as purine, uridine, and cytidine.

REVIEW OF LITERATURE

The principle of the method is that of the wet bulb thermometer. As early as 1913, A. W. Reed [17] demonstrated a lowering of vapor pressure of a solution by two wet bulb thermometers, washed with sodium chloride solution and water, respectively. Reed used ordinary thermometers and gave no quantitative results. The first development of a truly thermoelectric osmometer seems to be that of A. V. Hill[7] in 1930. Hill used a thermopile which, because of its size, required that the test solution be applied to a strip of filter paper, which was then placed directly upon the thermopile. This assembly was then inserted into a water chamber, thermostatted by a surrounding water bath, and approximately thirty to forty minutes were required before equilibrium could be established. Even with this crude arrangement, sensitivities of the order of a few milliosmoles could be obtained.

In 1934, E. J. Baldes[1], working in Hills laboratory, converted this to a true micro-method by using single thermocouple loops for holding the sample and reference solvent, although long reading times were still required. This type of device was used in a number of medical research laboratories in England, during the latter 1930's; an improved version of this thermocouple device was reported by V. E. Kinsey[10] in 1950. The following year, Brady, Huff, and McBain[2, 8] reported from Stanford Research Institute on the first version of a thermoelectric osmometer to use thermistors as the sensing elements. The higher sensitivity, the greater physical and chemical stability of these sensors seemed a great improvement over the thermocouples, and Müeller and Stolten[12] published a detailed study of their use for this purpose in 1953. Subsequent papers by Iyengar[9], Neumayer[13], Higuchi, *et al*[6], and Goyan and Reck[5] provided a



A—Foam
B—Aluminum block
C—Chamber
D—Syringe guide
E—Thermistor probe
F—Syringe in down (loading) position
G—Solvent cup and wick

Fig. 2

firm theoretical basis for the use of this device.

The instrument described here is a fully engineered design by Ehrmantraut, Marshall and Walker[15], from a prototype developed by Pasternak and Hiam[16] at Stanford Research Institute.

DESCRIPTION OF INSTRUMENT

As shown in Figure 2, the temperature control chamber consists of a hollow aluminum block wound with a resistance wire heater, which can be controlled with a mercury thermostat

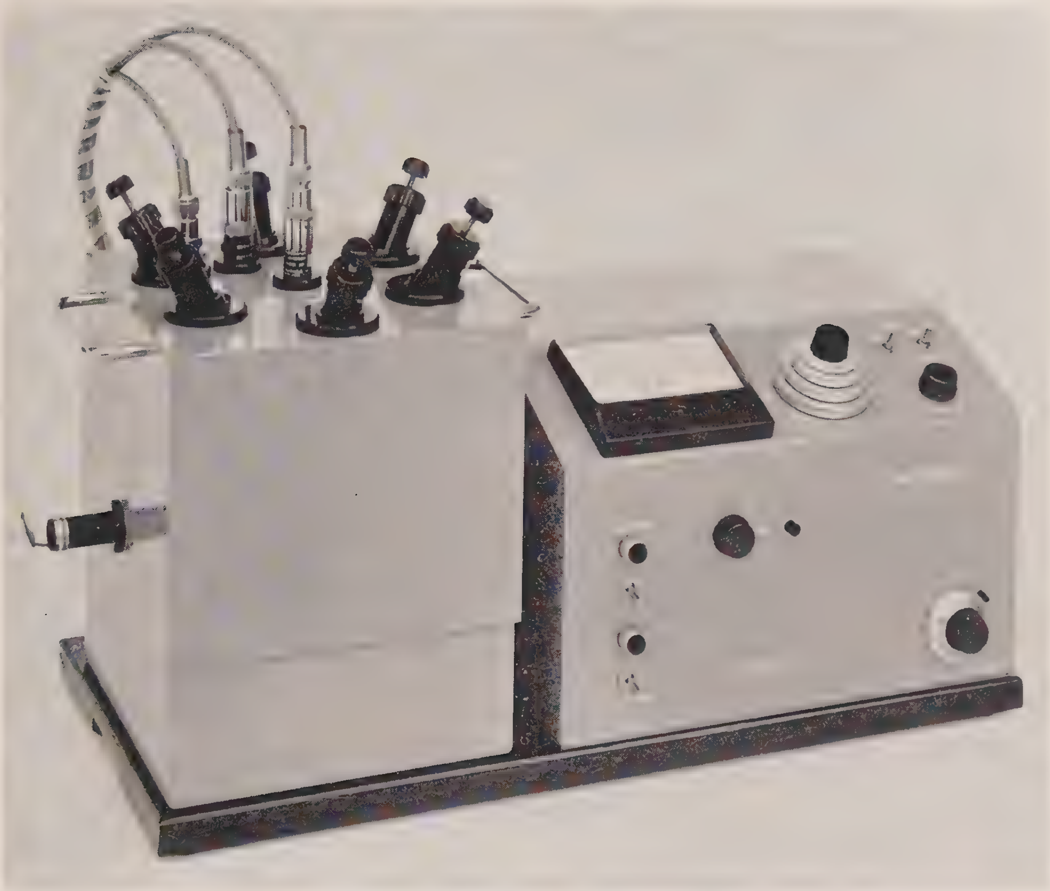


Fig. 3

so that the temperature in the vicinity of the sensing elements is held to better than ±.001° C. over a thirty-minute interval. Thermistors are mounted in the center of this chamber, and they are so positioned that hypodermic syringes containing solvent or samples may be lowered through guide tubes allowing the sample to flow over the thermistor bead, leaving a drop in place. Windows in the block allow observation of the samples during transfer. The thermal chamber is completely isolated with urethane foam from the surrounding atmosphere, and the prime design feature is the use of guide tubes with very small apertures so that outside air is not allowed into the stabilized chamber during the exchange of syringes and samples. The syringes (five for the sample bead and one for the reference bead) slide on rotation into the block to a fixed position, adjusted so that the needle tip almost touches the bead. After delivering a drop to the bead, the syringe is retracted; the thermostat is not disturbed; no special skill is required. A cup of solvent, with a specially-cut paper wick almost completely enclosing the thermistors, maintains a uniform vapor atmosphere in the thermal chamber. The sensing elements are two small bead thermistors having a resistance of about 6,000 ohms at 37° C., matched to better than 1 per cent, and with a temperature coefficient of 3.7 per cent. The special matching makes the measurements of resistance differences relatively insensitive to possible temperature fluctuations of the thermostat. The change in the thermistor resistance is measured with a simple D.C. resistance bridge, the sensitivity and stability of which permits determination of minimum increments of 0.01 ohm, which is equivalent to about 5 x 10^{-5} °C.

The electronics, which are housed in the right-hand part of the instrument shown in Figure 3, consists of a chopper-stabilized D.C. amplifier, especially sensitive to permit detection of changes of 0.2 microvolts. Actual measurements are taken from the decade box shown mounted on the sloped panel. The instrument is calibrated with known solutions of NaCl or mannitol, which is particularly useful since it is linear in milliosmol concentration over a very wide range. Following calibration, a graph is prepared showing resistance readings vs. milliosmol concentrations, and unknowns may be read directly.

STANDARD SOLUTIONS

Since temperature shift is a colligative effect, dependent solely upon the number of dissolved molecules and independent of their chemical characteristics, the instrument may be calibrated with a concentration series of a known solute. Unknowns in the same solvent may then be read directly from the calibration curve. Experience indicates that recalibration is not necessary unless, of course, the thermistor probe is replaced.

The vapor pressure osmometer may be calibrated with sucrose solutions as well as the same NaCl solution used to calibrate cryoscopes. Mannitol also makes a convenient calibrating material, because below 1,000 milliosmoles, there is less than 1 per cent departure from linearity, even if straight molal concentrations are used. Thus, if one needs a special calibration concentration, say 287 milliosmol per L., preparing the comparable molal solution (0.287 molal) will give excellent results. Values to be used in making calibration mixtures are given in Table I.

TABLE I

MILLIOSMOLES	NaCl CONC. g/1000 H_2O	MANNITOL g/1000 H_2O
100	3.089	18.22
200	6.252	36.44
300	9.457	54.66
400	12.702	72.88
500	15.93	91.1
750	24.10	136.5
1000	32.23	182.2
1500	47.97	
2000	64.37	
3000	95.40	
4000	125.2	
5000	153.9	
6000	180.8	
7000	207.0	
8000	230.5	

SPECIAL APPARATUS

A Mechrolab Model 301A Vapor Pressure Osmometer, equipped with 37° C. thermostat and aqueous thermistor probe is required. This unit will measure osmolalities of plasma, serum, or urine samples of 0.1 ml. up to values of about 1,800 mOs per L. Special accessories useful for research include an extra decade to permit measurements to about 8,000 mOs per L., and special

Hamilton microsyringes to allow measurements on 15-microliter samples[4].

PROCEDURE

The following is a summary of the operating procedure. Additional details can be obtained from the manufacturer's operating instruction.

1. Draw approximately 0.1 ml. of serum into syringe, place in holder, and insert into thermal block for pre-equilibration. Four different samples may be inserted simultaneously. Syringes Nos. 5 and 6 should be filled with distilled water.

2. After a few minutes, for thermal equilibration, syringes Nos. 5 and 6 are lowered, the thermistor beads rinsed and drops of distilled water left in place.

3. After one minute, zero the amplifier.

4. At two minutes, adjust the thermistor bridge with the balance control so that the meter is at zero.

5. Lower the No. 1 syringe, rinse several drops of serum sample across the sample bead, and leave a drop of serum in place on the thermistor.

6. At two minutes following placement of the serum drop, adjust the decade resistance box until the meter needle has returned to zero and record ΔR reading.

7. Lower the next syringe and make measurements in the same manner on the next sample.

8. Using the ΔR values from the calibration graph, record the serum osmolalities.

SOURCES OF ERROR

1. Errors can be introduced through the use of contaminated water. Presence of an unknown solute in the solvent will cause a lower temperature differential, resulting in lower than normal milliosmolality.

2. Insufficient rinsing of the previous sample drop can cause cross contamination resulting in erroneous results. Careful rinsing will eliminate this error.

3. Occasionally contamination of the reference drop can occur from splashing when placing a drop on the measuring bead.

Note: Errors from these sources can be detected and eliminated by checking for thermometric balance when solvent is placed on both beads.

4. If the serum protein is allowed to denature, erroneous osmolalities will result due to changes in the colligative properties.

RANGE OF VALUES

The blood serum of normal humans shows a typical osmolality of 285 to 295 mosm.[11, 14, 18]. This appears to be sistent in male or female, adult or child.

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Flame Photometric Method for Serum Cations

(Sodium, Potassium, Calcium and Magnesium)

HENRY A. TELOH, M.D.

INTRODUCTION

Qualitative identification of elements by analysis of flame spectra was successfully carried out over 100 years ago when Kirchoff and Bunsen, in 1860, reported the use of flame analysis for detection of various elements. Little use was made of this observation until the appearance of the work of Lundegardh, who published his first treatise on the quantitative estimation of intensity of flame spectra in 1929.

Lundegardh used a spectrographic technic for quantitation of flame spectra. An unknown solution was atomized into a flame placed in front of a spectrograph. After sufficient exposure, the photographic plate was developed, and the intensity of various spectral lines was compared with intensity of spectral lines of solutions of known composition. Optical density readings of lines on the photographic plate could then be correlated with concentration of the substance concerned with reasonable accuracy. This method obviously entailed considerable manipulation and delay, with serious limitation in accuracy due to difficulties in obtaining photographic emulsions of reproducible quality.

Because of technical difficulties inherent in the flame spectrographic method, direct-reading flame photometers were developed in Germany as early as 1935. In 1945, there was described the first direct-reading instrument in this country. A phototube was utilized to measure intensity of light emitted by spectral lines, obviating the use of photographic plates with their inherent difficulties and inaccuracies. By 1947, commercially available instruments appeared, and flame photometry was demonstrated to be a clinically useful method in the estimation of sodium and potassium in biological fluids. During the past decade, there has been a tremendous increase in popularity of the method and this is due principally to ease of performance and ready availability of results for clinical diagnostic work.

THEORY OF EMISSION PHOTOMETRY

Emission photometry, in contrast to absorption photometry, involves measurement of intensity of light emitted by a substance under certain conditions. Many substances, when subjected to thermal excitation of sufficient intensity, emit light of a characteristic wave length. Identification of the wave length of emitted light may be used in qualitative identification of the substance or substances concerned. Conversely, the magnitude of emitted light at a given wave length is, within specified limits, a function of concentration of element present.

Intense thermal excitation of atoms results in displacement of orbital electrons from their normal position, a process which is associated with absorption of thermal energy. Since displacement of orbital electrons results in instability of the atom, there follows replacement of electrons to the normal orbital positions. This replacement of orbital electrons results in evolution of radiant energy in the form of light in the visible portion of the spectrum. The wave length of the emitted light is determined by the energy level of orbital electrons displaced and is characteristic for each element.

Spectra available for analysis are called line and band spectra. Line spectra are produced by thermal excitation of electrons of the atomic form of an element. Line spectra are narrow and sharply defined. Many line spectra (e.g. sodium, potassium, lithium) are quite intense, but this is not universally true. Band spectra or "oxide bands" are the result of thermal excitation of the oxide form of the element. They are invariably wide and are frequently not as intense as line spectra.

Estimation of Sodium

PRINCIPLE

Sodium produces an atomic spectral line with intense light emission at 589.3 mμ. A second line at 330.2 mμ is of no importance because of very low intensity of emitted light.

Either a direct-reading or internal standard type of flame photometer may be used for quantitation. In the direct-reading type of procedure, intensity of emitted light of solutions of unknown concentration is directly compared to intensity of emitted light of solutions of known concentration. Calibration curves for sodium are not linear and must be constructed by using concentration of standard solutions enclosing the unknown.

In the internal standard procedure, the intensity of emitted light of the unknown is compared to the intensity of emitted light of an arbitrarily included element (e.g., lithium) which is present in the same concentration in both standard and unknown solutions.

The procedures described in this chapter refer to use of the Beckman Model B spectrophotometer with flame attachment.

STANDARD SOLUTIONS

1. *Sodium Stock Standard Solution.* A stock standard solution is prepared

by placing 8.1830 grams of dessicated sodium chloride in a 1-L. volumetric flask and diluting to volume with distilled water.

2. *Sodium Working Standard Solutions.* Ten, 15, 20, and 25 ml. of sodium stock standard solution are pipetted into 1,000-ml. volumetric flasks. To each volumetric flask are added 20 ml. of a standard solution containing 4.0 mEq. per L. of potassium, 5.0 mEq. per L. of calcium, 2.0 mEq. per L. of magnesium, 4.0 mg. per 100 ml. of phosphorus, 100 mg. per 100 ml. of glucose, and 15 mg. per 100 ml. of urea expressed as urea nitrogen. The contents are diluted to the mark with distilled water. These working standards represent 70, 105, 140, and 175 mEq. per L. of sodium in serum when the serum is diluted 1:50.

SPECIAL APPARATUS

Beckman Model B. spectrophotometer with flame attachment at wave length 589.3 mμ; blue sensitive phototube; sensitivity switch in 4 position. Oxygen-hydrogen, oxygen-acetylene or oxygen-propane fuel may be used.

PROCEDURE

1. A 1:50 dilution of serum sample is prepared.

2. The shutter is kept open during the entire procedure. While aspirating the 70 mEq. per l. standard (low standard), the galvanometer needle is adjusted to zero per cent transmission by means of the dark current control. While aspirating the 175 mEq. per L. standard (high standard), the galvanometer needle is adjusted to 100 per cent transmission by means of the slit width control. Since these two settings are interdependent, the dark current and slit width adjustments are alternately corrected until the low standard reads zero and the high standard reads 100 per cent transmission. The readings of the intermediate standards and of the unknown serum are then determined.

3. A calibration curve is constructed by plotting per cent transmission readings on the abscissa and concentration of standards on the ordinate. The concentration of the unknown is read from the calibration curve.

DISCUSSION

This is the recommended procedure for estimation of sodium using the Beckman instruments and Coleman flame photometer. A curve can be constructed with a spread of about one milliequivalent of sodium per scale division. This permits use of full scale deflection on the transmission scale. Since wider slit widths must be used, there is somewhat greater needle instability or background noise than with previous methods. The standard deviation of multiple analyses made on a control serum done under routine conditions of analysis is about ± 1.0 mEq. per L.

SOURCES OF ERROR

1. The interfering effect of other ions in the serum is negligible in the case of sodium because the concentrations of these ions normally are small.

2. The water used for solutions and for dilutions should be distilled water or deionized water of high grade. Ideally, the same water should be used in making up all standard and unknown dilutions.

3. Leaching effect may be of import-

ance when glass containers made of soft glass are used. All standards should be stored in hard glass containers or preferably in polyethylene containers.

4. All working standard solutions and dilutions of samples to be analyzed must be a uniform temperature and preferably at ambient temperature. If standards are stored in the refrigerator, they must be brought to room temperature before using them.

5. Calibration of glassware. Volumetric glassware used in preparing standards and sample dilutions in flame photometry should be of the best quality. Minor changes in sodium concentration of serum which may be clinically meaningful may be caused by errors in dilution produced by inferior glassware.

6. Care of glassware. All glassware must be chemically clean. Contamination with sodium may occur as a result of exposure to a dusty atmosphere or an atmosphere loaded with vapor containing sodium. Sweat from the skin transferred to glassware by contact may introduce serious error.

RANGE OF VALUES –

The normal range of values is given in Table I.

TABLE I NORMAL CONCENTRATIONS FOR SERUM SODIUM

AUTHORS	YEAR	METHOD	NORMAL VALUES mEq. PER LITER
Elliott and Holley	1951	Flame Photometry	Range 135-155 Mean 144.7 S.D. 3.81
Smith *et al.*	1950	Spectrography	Range 136-158 Mean 142.0
Marinis	1947	Flame Photometry	Range 135.5-153.2 Mean 144 S.D. 3.6
Overman	1947	Flame Photometry	Range 140-159 Mean 153
Hald	1947	Chemical Analyses	Range 130-145
Smith	1950	Flame Photometry	Range 136-158 Mean 142
Wynn	1950	Flame Photometry	Range 139-152 Mean 144.5
Kramer and Tisdall	1921	Pyroantimonate Method	Range 140.4-152.1 Mean 146.2
Rourke	1928	Pyroantimonate Method	Range 143.9-150.8 Mean 147.3
Sunderman	1930	Gravimetric Method	Range 133.6-137.3 Mean 135
Darnell	1940	Colorimetric Method	Range 144.8-151.3 Mean 148.2
Albanese	1948	Colorimetric Method	Range 133-141

Estimation of Potassium

PRINCIPLE

The principles of analysis are similar to those given in the section on estimation of sodium. While not as intense as the spectral line of sodium, the 767 mμ. line of potassium is sufficiently intense to pose no problem in quantitation. A spectral line occurring at 404.6 mμ. is of no practical value because of its very low intensity.

STANDARD SOLUTIONS

1. *Stock Standards.* A stock standard solution of potassium is prepared by placing 0.2982 gm. of dessicated potassium chloride in a 1,000-ml. volumetric flask and diluting to volume with distilled water. This stock standard solution contains 4.0 mEq. per L. of potassium. A stock standard solution of sodium is prepared by placing 8.1830 gm. of dessicated sodium chloride in a 1,000-ml. volumetric flask and diluting to volume with distilled water. This stock standard solution contains 140 mEq. per L. of sodium.

2. *Working Standard Solutions.* Ten, 20, 30, and 40 ml. of potassium stock standard solution are added to 1,000-ml. volumetric flasks. To each flask are added 20 ml. of sodium stock solution. The contents are diluted to the mark with distilled water. These solutions represent 2, 4, 6, and 8 mEq. per L. of potassium in serum when the serum is diluted 1:50. Each potassium working standard contains the equivalent of 140 mEq. per L. of sodium.

SPECIAL APPARATUS

Beckman model B spectrophotometer with flame attachments with red sensitive phototube at wave length 767 mμ. Oxy-hydrogen, oxy-acetylene or oxy-propane flame may be used. Sensitivity switch is set in 4 position.

PROCEDURE

1. A 1:50 dilution of the serum sample is made.

2. With the shutter control open and while aspirating distilled water, the galvanometer needle is adjusted to zero per cent transmission by means of the dark current control. While aspirating the middle (4.0 mEq. per L.) standard, the galvanometer needle is adjusted to 40 per cent T. These two settings are repeated alternately for fine adjustment. Under identical operating conditions, the per cent T. readings of the high and low standards and unknown serum are determined and recorded.

3. Since the calibration curve is, for practical purposes, linear at the dilutions used here, the concentration of potassium in the sample is easily determined by dividing the per cent T. by ten. Practically, the spectrophotometer can be calibrated with only one standard since the curve is linear.

DISCUSSION

Once the instrumental settings have been determined by calibration with a standard solution, concentrations of potassium in the unknown samples can be determined very rapidly, since they can be read off directly from the per cent T. scale without the necessity of constructing a calibration curve.

The standard deviation achieved on multiple analyses of a control serum under routine conditions of analysis is about ±0.05 mEq. per L.

SOURCES OF ERROR

In general, the sources of error in analysis of serum for potassium are those inherent in flame photometry and are listed in the discussion of sodium analysis. The interference, in terms of potentiation, produced by the presence of sodium is of some importance. It is for this reason that working potassium standards should contain a concentration of sodium approximating that of serum.

RANGE OF VALUES—

The normal range of values is given in Table II.

TABLE II NORMAL CONCENTRATIONS OF SERUM POTASSIUM

AUTHORS	YEAR	METHOD	NORMAL VALUES mEq. PER LITER
Elliott and Holley	1951	Flame Photometry	Range 3.1-5.5 Mean 4.18 S.D. 0.43
Smith	1950	Spectrography	Range 3.61-4.85 Mean 4.06
Marinis	1947	Flame Photometry	Range 3.6-6.2 Mean 4.52 S.D. 0.45
Overman	1947	Flame Photometry	Range 3.6-7.2 Mean 5.1
Hald	1947	Chemical Analyses	Range 4.2-4.9
Smith	1950	Flame Photometry	Range 3.6-4.8 Mean 4.1
Wynn	1950	Flame Photometry	Range 3.7-5.1 Mean 4.5
Faber	1951	Flame Photometry	Range 3.7-5.3 Mean 4.4 S.D. 0.36
Kramer and Tisdall	1921	Cobalt, Titration	Range 4.6-5.5 Mean 5.0
Atchley	1923	Chloroplatinate Method	Range 3.1-5.2
Lockhead	1951	Colorimetric Method	Range 3.7-5.7 Mean 4.8
Wilhelm	1951	Silver Cobalt Method	Range 4.2-5.3
Danowski	Unpubl.	Flame Photometry	Infants 5.9 ±1.4 Children 4.3 ±0.4 Young Adults 4.4 ±0.3 Old Age 4.6 ±0.4

Estimation of Calcium

PRINCIPLE

There are three wave lengths available for quantitation of calcium. A sharply defined atomic line occurs at 422.7 mμ, with two wide molecular oxide bands at 556 mμ and at 626 mμ. All these bands or lines are satisfactory for analysis, although the optimal wave length is determined by individual characteristics of the instrument employed.

Low intensity of spectral emission of calcium, as compared to sodium and potassium, requires high photodetector sensitivity and relatively low dilution for adequate quantitation. High photodetector sensitivity is achieved on the

Beckman spectrophotometers by means of a photomultiplier circuit.

Calibration curves are linear at all wave lengths and permit direct reading of results on the transmission dial. A calcium standard serum is used, the calcium content of which has previously been determined. A calibration curve is constructed, the zero point of which is represented by the luminosity of a blank solution composed of sodium in a concentration of 140 mEq. per L. The instrument is calibrated by setting the galvanometer needle at a reading corresponding to concentration of calcium in the serum standard times 10 on the per cent T. scale.

REAGENTS

A blank solution is used for the zero adjustment of the flame background. It consists of a 1:25 (v:v) dilution of a solution containing 140 mEq. per L. of sodium.

STANDARD SOLUTION

A 1:25 (v:v) aqueous dilution of calcium standard serum is prepared. A control serum of known calcium concentration or a pooled serum, the calcium concentration of which has been determined by chemical methods, may be used.

SPECIAL APPARATUS

Beckman Model B spectrophotometer with flame accessories and photomultiplier phototube with wave length settings at 422.7, 556 or 626 mμ; selector switch at 4 and photomultiplier setting at full sensitivity. Oxyhydrogen, oxyacetylene or oxypropane fuel may be used.

PROCEDURE

1. A 1:25 (v:v) dilution of the unknown serum sample is prepared.
2. While aspirating the serum standard and by varying the slit width control, the galvanometer needle is adjusted to the per cent. T. reading corresponding to the concentration of calcium in the serum standard multiplied by ten.
3. While aspirating the blank solution, the galvanometer needle is adjusted to zero per cent T. by varying the dark current control.
4. These two settings are interdependent and must be alternately adjusted until the blank solution reads zero per cent T. and the standard reads calcium concentration of standard times ten.
5. The diluted unknown serum is then introduced into the flame, and the galvanometer reading is recorded. The calcium concentration in the serum is calculated by dividing the per cent T. reading by ten.

DISCUSSION

Reproducibility of the method is good with a standard deviation of replicate samples of about 0.05 mEq. per L.

SOURCES OF ERROR

Sodium exerts a significant augmenting effect on the spectral emission of calcium. This effect is, in great part, offset by using a blank solution containing 140 mEq. per L. of sodium. Where wide variations of sodium concentration occur, errors in estimation of calcium of up to ±5.0 per cent will be encountered.

Phosphorus has a depressing effect on emission of calcium. As an example, an increase of phosphorus from 4.0 to 5.0 mg. per 100 ml. of serum results in apparent decrease in serum calcium from 5.0 to 4.65 mEq. per L., an error of seven per cent. Variations in concentration of serum phosphorus will, therefore, introduce significant error in results obtained by flame photometric methods.

RANGE OF VALUES

Normal values obtained by flame photometry are slightly, but significantly, higher than those obtained by the oxalate precipitation and permanganate titration methods. The average deviation between the two methods is about 0.22 mEq. per L. of calcium.

Estimation of Magnesium

PRINCIPLE

Three wave lengths are available for analysis of magnesium by flame photometry. A spectral line at 285.2 mμ. is in the ultraviolet range and is not ordinarily used. Two spectral bands of the oxide form of the element occur at 370.8 and 383 mμ. These bands have an intensity of emitted light approximately 0.1 per cent that of sodium. Because of this low intensity of emitted light, quantitation by flame photometry poses difficult technical problems and requires high photodetector sensitivity available only in the photomultiplier type of phototube. Despite high phototube sensitivity, relatively wide slit widths must be used intensifying interference effect of other ions. Low sample dilution also complicates technical procedures because of sample viscosity and likelihood of plugging of the atomizer assembly. For this reason, most methods require extensive chemical pretreatment for destruction of serum proteins. This then permits analysis of samples of relatively high concentration of magnesium.

The method presented here is a self-standardizing method in which the luminosity value of a known quantity of magnesium under the conditions of interference present in the unknown serum is determined by measuring the increase in luminosity produced by addition of a standard amount of magnesium to the serum. The magnesium concentration in the unknown serum is then determined from the ratio of luminosity of the unknown to luminosity of the known quantity of magnesium.

SPECIAL APPARATUS

Beckman, Model B spectrophotometer with flame attachment; oxyhydrogen flame; photomultiplier phototube at full sensitivity; shutter control in the up position with blue filter; slit width 0.3 mm. Background luminosity is measured at 360 mμ: luminescence due to magnesium is measured at 371 mμ.

PREPARATION OF STANDARD AND SPECIMEN

To each of two 10-ml. volumetric flasks labelled “A” and “B” is added 1 ml. of serum. To flask B is added 1 ml. of an aqueous solution containing 2 mEq. per L. of magnesium. The contents of each flask are diluted to the mark with distilled water or with 0.1 per cent *Sterox*.

PROCEDURE

1. While aspirating the 1:10 dilution of serum A, with the shutter open (up position), and with the wave length setting at 360 mμ, the meter needle is adjusted to zero per cent T. by varying the dark current control. The wave length setting is then adjusted to 371 mμ. The per cent T. reading of serum (A) is determined. The per cent T. reading of the 1:10 dilution of serum plus magnesium standard (B) is then determined under identical operating conditions at 371 mμ.

2. Calculation:

$$\frac{\%\text{T of A.}}{\%\text{T of }(\text{B} - \text{A})} \times \text{Mg standard in mEq. per L.} = \text{serum conc. in mEq. per L.}$$

DISCUSSION

Because of low serum concentrations and low intensity of spectral emission, quantitation of magnesium by flame photometry requires careful and precise technic. Since the limits of capability of the detection system are approached, the method requires careful attention to optimal functioning of the spectrophotometer with exact adjustment of wave length settings, wave length calibration, reflecting mirrors, and optical system. Oxygen and fuel adjustments must be carefully made to insure optimal emission of light.

A calibration curve established by adding increasing amounts of magnesium to serum B results in a straight line curve with concentrations up to 10 mEq. per L. of magnesium.

Analysis of replicate specimens yields a standard deviation of ±0.06.

RANGE OF VALUES

Using the self-standardizing method, the average normal value for serum magnesium was 2.04 mEq. per L. with a range of 1.80 to 2.30.

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Atomic Absorption Spectrometry of Calcium and Magnesium in Biological Fluids

F. WILLIAM SUNDERMAN, JR., M.D., and JOHN E. CARROLL, B.S.

In 1860, Kirchoff and Bunsen[15] observed that the wave lengths of the dark Fraunhofer absorption lines in the solar spectrum coincided with the wave lengths of elemental lines in various emission spectra. Based upon the assumption that atoms absorb light at the same wave lengths as they emit light, their observation revealed the presence of several elements in the solar atmosphere.

In 1955, Walsh[25] showed that the phenomenon of atomic absorption could serve as a spectrochemical basis for the quantitative determination of metals. He demonstrated that measurements of metals by atomic absorption spectrometry are more sensitive than measurements by flame emission spectrometry, and less subject to interference from other elements[25].

Within the past decade, more than one hundred papers have been published which describe analytical applications of atomic absorption spectrometry. The recent literature on atomic absorption has been comprehensively reviewed by Allan[3], David[8], Elwell and Gidley[10], Robinson[21], Willis [31], and Zettner[32]. The theoretical and instrumental factors which influence quantitative determinations of metals by atomic absorption spectrometry have been summarized by Allan[2], Fuwa and Vallee[11], Malmstadt and Chambers[17], Menzies[18], Millazo[19], and Russell, Shelton and Walsh[22].

An atomic absorption method for the determination of calcium in biological materials was reported in 1960 by Willis[27, 28, 30], and has been modified by Newbrun[20] and by Zettner and Seligson[33]. Use of atomic absorption spectrometry for the determination of magnesium in biological materials was reported in 1959 by Willis[26, 29, 30], and has been modified by Dawson and Heaton[9] and by Horn and Latner[13]. A further modification of the atomic absorption procedures was developed by Sunderman and Carroll[24] which enables precise determinations of calcium and magnesium to be performed upon a single dilution of a protein-free filtrate of serum.

The atomic absorption spectrometer which is employed in our laboratory is illustrated schematically in Figure 1.

SCHEMATIC DIAGRAM OF ATOMIC ABSORPTION SPECTROMETER

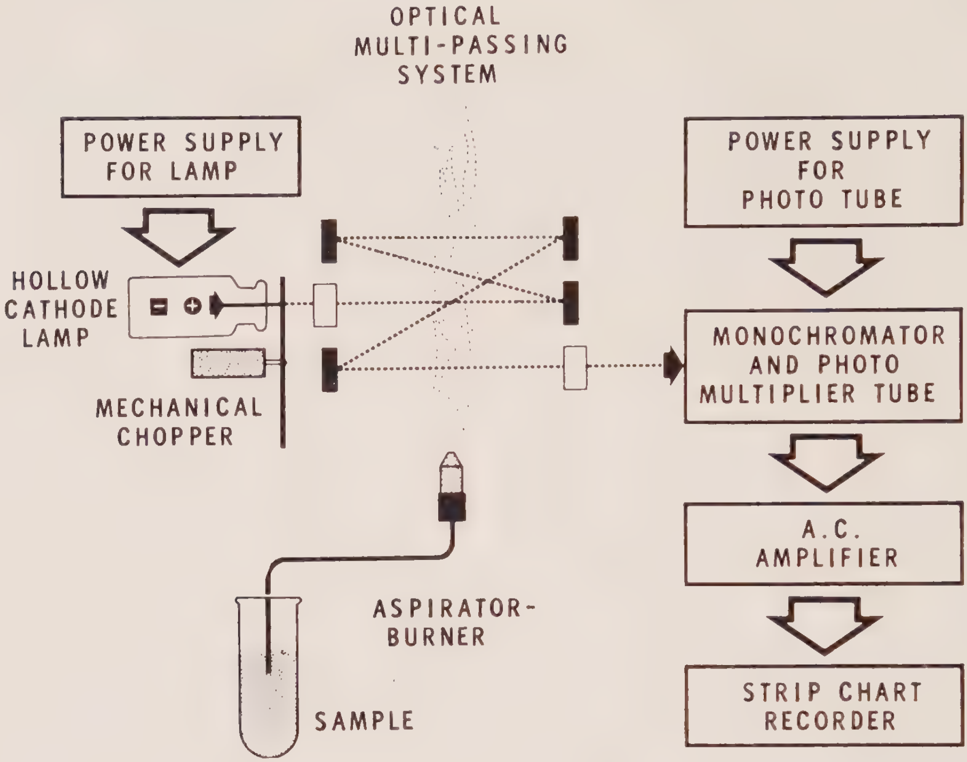

Fig. 1

Aspiration of a protein-free filtrate of serum into the burner produces thermal molecular dissociation and dispersion of calcium and magnesium atoms throughout the flame. Small proportions of these atoms become excited to emit light, but the overwhelming majorities of the atoms remain in the ground state and are capable of absorbing discrete wave lengths of incident light. These specific wave lengths are provided by a lamp with a hollow cathode constructed of an alloy containing both calcium and magnesium. The beam of light is passed several times through the flame, and it is then focused upon the entrance slit of a diffraction grating monochromator. The absorptions of light at the specific wave lengths are proportional to the concentrations of calcium and magnesium in the sample.

The multi-passing optical system increases the optical path length within the flame, thereby enhancing the sensitivity of the atomic absorption technic. This system also minimizes fluctuations in atomic absorption which may result from variable distribution of ground-state atoms within the flame[18, 26]. The abundance of atoms in the ground-state, in comparison to those in the excited state, accounts for the greater sensitivity of atomic absorption spectrometry relative to flame emission spectrometry.

Light which is emitted from the

flame constitutes a potential source of error in atomic absorption spectrometry, since the light energy which strikes the photomultiplier tube represents the net balance of emission and absorption. This source of error is avoided by modulating the incident light beam with a mechanical chopper, and tuning the photomultiplier detector circuit to the same frequency of modulation. Under these conditions, the detector circuit responds only to the pulsed signal from the light beam, and does not respond to the continuous signal produced by light emission from the flame[25]. The alternating current from the photomultiplier detector circuit is amplified and recorded.

Method for Determination of Calcium and Magnesium in Serum [24]

REAGENTS

1. *Strontium Chloride Solution in 0.2 N Hydrochloric Acid.* Into a 1-L. volumetric flask are transferred 15.2 gm. of strontium chloride ($SrCl_2 \cdot 6H_2O$). Concentrated hydrochloric acid, 17.5 ml., is added to the flask. The contents are dissolved in distilled water and diluted to the mark. Each lot of strontium chloride reagent should be tested to verify that it is not contaminated with calcium or magnesium.

2. *Sodium Chloride Solution* (70 mEq. per L.). Into a 1-L. volumetric flask are transferred 4.09 gm. of sodium chloride, reagent grade. The contents of the flask are dissolved in distilled water and diluted to the calibration mark.

3. *Trichloracetic Acid Solution,* 10 per cent (w/v).

4. *Trichloracetic Acid Solution,* 22.5 per cent (w/v).

5. *Calcium Stock Standard Solution* (10 mg. of calcium per 100 ml.). Reagent grade calcium carbonate is dried at 120° C. for four hours and then cooled in a desiccator. Into a 1-L. volumetric flask is placed 0.2498 gm. of calcium carbonate. Ten ml. of 1.0 N hydrochloric acid are added, and after the calcium carbonate has dissolved, the contents of the flask are diluted with water and mixed. With the temperature at 25° C., the volume is adjusted to the calibration mark.

6. *Calcium Working Standard Solutions.* Into 50 ml.-volumetric flasks are transferred 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 ml of calcium stock standard solution. Twenty-five ml. of strontium chloride solution, 5 ml. of sodium chloride solution, and 10 ml. of 22.5 per cent trichloracetic acid solution are added to each flask, and the contents are diluted to the mark with distilled water. These standard solutions are equivalent to 0, 2, 4, 6, 8, 10, 12, and 14 mg. of calcium per 100 ml. of serum.

7. *Magnesium Stock Standard Solution* (2 mg. of magnesium per 100 ml.). Into a 1-liter volumetric flask is placed 0.2027 gm. of magnesium sulfate ($MgSO_4 \cdot 7H_2O$). The contents of the flask are diluted with water and mixed. With the temperature at 25° C., the volume is adjusted to the mark.

8. *Magnesium Working Standard Solutions.* Into 50-ml. volumetric flasks are transferred 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 ml. of magnesium stock standard solution. Twenty-five ml. of strontium chloride solution, 5 ml. of sodium chloride solution, and 10 ml. of 22.5 per cent trichloracetic acid solution are added to each flask, and the contents are diluted to the mark with distilled water. These standard solutions are equivalent to 0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, and 2.8 mg. of magnesium per 100 ml. of serum.

ATOMIC ABSORPTION APPARATUS

A Jarrell-Ash atomic absorption apparatus (Model 82-362) is employed with the following modifications:

1. A Westinghouse hollow cathode discharge lamp (Model WL-22604) is used for measurements of both calcium and magnesium.
2. A Zeiss hydrogen-air burner (Model 50-56-23) is mounted at the midpoint of the optical path with the tip of the burner 14 cm. from the top of the optical bench. The canula of the burner is attached to a 6-cm. length of polyethylene tubing, so that the sample may be aspirated from a test tube. Polyethylene tubing with an inside diameter of 0.030 in. is satisfactory (Clay-Adams tubing #PE 60).
3. The output of the photomultiplier tube detector circuit is recorded by means of a 10 m.v. strip-chart recorder (Honeywell Regulator Co., Model #E15-1).

PROCEDURE

Preparation of Sample Into a 12-ml. centrifuge tube are transferred 0.5 ml. of serum and 4.5 ml. of 10 per cent trichloracetic acid. The contents of the tube are mixed with a "Vortex" rotary mixer and are allowed to stand for ten minutes. The tubes are centrifuged at 2500 r.p.m. for fifteen minutes. Three ml. of the clear supernatant are transferred to a test tube. Three ml. of strontium chloride solution are added, and the contents of the test tube are mixed with a "Vortex" mixer.

Adjustment of the Atomic Absorption Apparatus The power supply to the calcium-magnesium hollow cathode lamp is adjusted to 20 m.a. The beam of light from the hollow cathode lamp is focused upon the monochromator entrance slit. The monochromator is adjusted to measure the absorption at the wave lengths specified for calcium or magnesium in Table I. The air regulator valve is opened first, followed by the hydrogen regulator valve, and the burner is ignited. The gas regulator valves are adjusted to provide the gas pressures specified in Table I for determinations of calcium or magnesium.

An opaque card is placed in front of the monochromator entrance slit, and the recorder is adjusted to 100 per cent absorption. The card is removed, and the recorder is adjusted to 0 per cent absorption while water is aspirated through the burner. Fluctuations in the base line are minimized by use of the damping adjustment. Stability of the burner, optical, and electronic systems is verified by monitoring the base line while water is aspirated through the burner.

Measurements of Atomic Absorption The polyethylene tubing connected to the burner is inserted into a test tube which contains one of the standards or samples. The absorption reading does

TABLE I OPERATING CONDITIONS FOR MEASUREMENTS OF SERUM CALCIUM AND MAGNESIUM BY ATOMIC ABSORPTION SPECTROMETRY

CALCIUM	
Sample Aspiration Rate	5.2 ml per min.
Hydrogen Pressure	15 lb. per sq. in.
Hydrogen Flow Rate	0.5 liters per min.
Air Pressure	15 lb. per sq. in.
Lamp Current	20 milliamperes
Absorption Wave Length	4227 Ångstroms
Entrance and Exit Slits	100 microns
MAGNESIUM	
Sample Aspiration Rate	3.0 ml per min.
Hydrogen Pressure	5 lb. per sq. in.
Hydrogen Flow Rate	0.3 liters per min.
Air Pressure	8 lb. per sq. in.
Lamp Current	20 milliamperes
Absorption Wave Length	2852 Ångstroms
Entrance and Exit Slits	100 microns

not become stable until the sample has been aspirated into the flame for two to three seconds. The atomic absorption of the sample is then recorded for five seconds. Immediately following the aspiration of each sample, water is aspirated through the burner until the absorption reading has returned to the base line. All measurements are performed in duplicate, and standard solutions of calcium or magnesium are analyzed with each group of samples.

At the conclusion of the analyses, water is aspirated through the burner for five minutes. The hydrogen regulator valve is closed, and the burner is dried by passing air through it for one to two minutes.

Calculations Measurements of per cent absorption (%A) are converted into absorbance (O.D.) units by the equation:

$$\text{Absorbance (O.D.)} = 2\text{-}\log_{10}(100 - \%A)$$

Calibration curves are used in computing the concentrations of calcium and magnesium in the samples of serum.

INSTRUMENTAL PARAMETERS

The absorption line of calcium at 4227 Å and the absorption line of magnesium at 2852 Å were used throughout these investigations. At these wave lengths, the optimum discharge current for the

TABLE II PRECISION OF MEASUREMENTS OF SERUM CALCIUM AND MAGNESIUM BY ATOMIC ABSORPTION SPECTROMETRY

	BECKMAN BURNERS	ZEISS BURNER	
CALCIUM			
Mean Concentration	10.0	10.0	mg/100 ml
Range:	9.3 − 10.7	9.8 − 10.2	mg/100 ml
Standard Deviation:	± 0.38	± 0.11	mg/100 ml
Coef. of Variation:	3.8	1.1	percent
MAGNESIUM			
Mean Concentration:	2.0	2.0	mg/100 ml
Range:	1.81 − 2.18	1.94 − 2.06	mg/100 ml
Standard Deviation:	± 0.10	± 0.03	mg/100 ml
Coef. of Variation:	5.1	1.6	per cent

TABLE III INTERFERENCES IN MEASUREMENTS OF SERUM CALCIUM AND MAGNESIUM BY ATOMIC ABSORPTION SPECTROMETRY

SOURCE OF INTERFERENCE	COMPENSATION FOR INTERFERENCE
SO_4 and PO_4 interference in determination of Ca	Addition of EDTA, Sr, or La to samples and standards
Na interference in determination of Ca	Addition of Na to standards
Protein interference in determination of Ca and Mg	Protein precipitation with trichloracetic acid, or addition of protein to standards

hollow cathode lamp was found to be 20 m.a. Measurements of calcium and magnesium were performed with monochromator entrance and exit slits of 25, 40, 50, and 100 micron widths. The greatest sensitivities were achieved with slit-widths of 100 microns.

Comparisons were undertaken of measurements of calcium and magnesium with fuel mixtures of hydrogen and air, acetylene and air, and acetylene and oxygen. The sensitivities which were obtained with a hydrogen-air flame were greater than those obtained with the other fuel mixtures. Moreover, the hydrogen-air flame provided the most stable base line and the least visual and auditory irritation.

The Jarrell-Ash atomic absorption spectrometer is manufactured with a burner manifold on which 3 Beckman hydrogen-air burners are mounted. The reproducibility of measurements of calcium and magnesium with the Beckman burners was found to be less than with a single Zeiss burner (Table 2). With use of the 3 Beckman burners, the coefficients of variation of 10 replicate measurements of serum calcium and magnesium were 3.8 and 5.1 per cent. When a single Zeiss burner was substituted for the Beckman burners, the coefficients of variation of 10 replicate measurements of serum calcium and magnesium were 1.1 and 1.6 per cent.

As shown in Figure 2, calibration curves with the Beckman burners and the Zeiss burner had similar configurations. As the slopes of the calibration curves were influenced by small fluctuations in gas pressure and in monochromator adjustment, it was necessary to prepare a calibration curve with each group of analyses.

SOURCES OF INTERFERENCE

Our observations regarding the sources of interference in measurements of serum calcium and magnesium, as summarized in Table 3, confirmed the findings of other workers.[1, 7, 9, 13, 16, 28, 29, 31] Phosphate and sulfate produced diminutions in the atomic absorption of calcium owing to the formation of calcium salts which are resistant to thermal molecular dissociation.[8, 12] Interference from sulfate and phosphate may be prevented by additions of EDTA (ethylenediamine tetraacetic acid), strontium, or lanthanum.[3, 24, 26] EDTA prevents interference by binding calcium and thus avoiding the combination of calcium with sulfate and phosphate.[20] Strontium and lanthanum prevent interference by uniting preferentially with the sulfate and phosphate ions.[20] Strontium and lanthanum chlorides were added to serum filtrates and to standard solutions in final concentrations of 2,500, 5,000, and 10,000 parts per million of Sr. or La. Under the conditions described for the procedure, additions of strontium or lanthanum in final con-

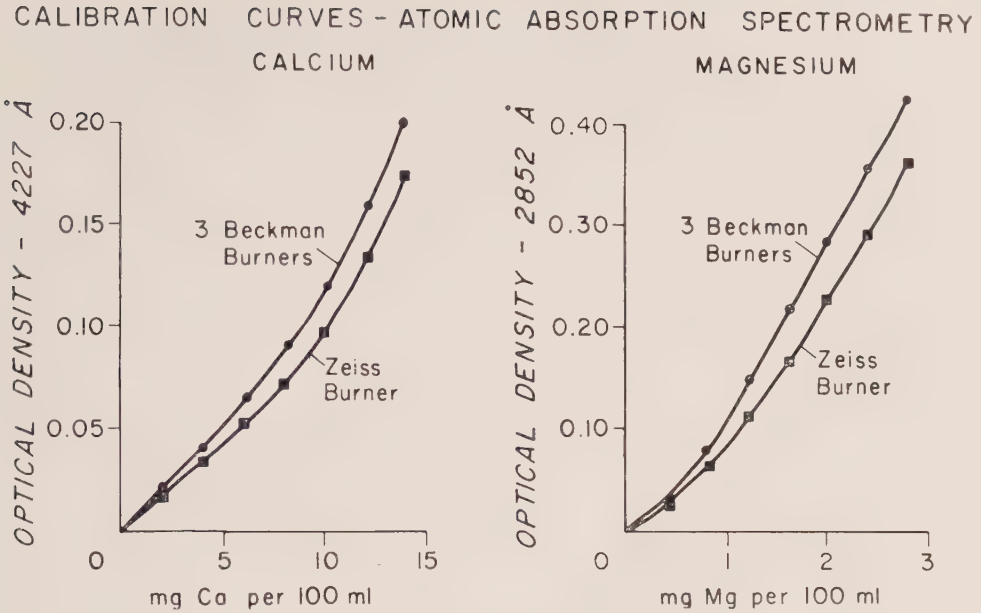

Fig. 2

centrations of 2,500 p.p.m. prevented interference from sulfate and phosphate throughout the ranges of concentrations encountered in pathologic serums. Strontium was found to have the special advantage that the red color of its emission spectrum facilitated visual monitoring of the performance of the burner.

Under the operating conditions, serum sodium produced a mean enhancement of 3 per cent in the atomic absorption of serum calcium. This source of error was avoided by the addition of sodium to the standard sample in a concentration equivalent to 140 mEq. per L. of serum. Sodium was not found to have a significant effect upon the atomic absorption of magnesium. Additions of potassium to the standard solutions in concentrations of 5 and 10 mEq. per L. did not influence the atomic absorption of calcium or magnesium. Additions of magnesium to the calcium standards in concentrations of 2 and 4 mg. per 100 ml., and additions of calcium to the magnesium standards in concentrations of 10 and 20 mg. per 100 ml. were likewise without effect.

Serum proteins produced inhibition in the atomic absorption of both calcium and magnesium. To avoid protein interference, Newbrun[20] recommended the analysis of protein-free filtrates, while Zettner and Seligson[33] recommended the preparation of calcium-free serum proteins by ion-exchange and dialysis, and addition of the decalcified proteins to the standard solutions in a concentration of 6.5 grams of protein per 100 ml. In our experience, preparation of trichloracetic acid filtrates of serum proved to be the most practical means of avoiding protein interference.

RECOVERY STUDIES

Measurements of the recovery of calcium and magnesium added to serum are listed in Table IV. Calcium stock standard solution was added to ten samples of serum in order to increase the concentrations of calcium by 4 mg. per 100 ml. Magnesium stock standard solution was added to eight samples of serum in order to increase the concentrations of magnesium by 0.8 mg. per 100 ml. As shown in Table IV, the recovery of calcium averaged 100 per cent with a range from 94 to 105 per cent. The recovery of magnesium averaged 102 per cent, with a range from 96 to 107 per cent.

TABLE IV MEASUREMENTS OF RECOVERY OF CALCIUM AND MAGNESIUM ADDED TO SERUM

Recovery of calcium added to 10 serums in concentration of 4 mg per 100 ml.	
Mean recovery	100 per cent
Range	= 94-105 per cent
Coefficent of variation	= 4.3 per cent
Recovery of magnesium added to 8 serums in concentration of 0.8 mg per 100 ml.	
Mean recovery	102 per cent
Range	= 96-107 per cent
Coefficient of variation	= 4.3 per cent

Comparisons with other methods

In Figure 3 determinations of serum calcium by atomic absorption spectrometry are contrasted with measurements by the fluorometric *EDTA* procedure of Kepner and Hercules[14]. In analyses of fifty serums using the Beckman burners, the slope of the regression line was 0.99, and the standard error of estimate was ± 0.39 mg. of calcium per 100 ml. In analyses of nineteen serums using the Zeiss burner, the slope of the regression line was 0.99, and the standard error of estimate was ± 0.23 mg. of calcium per 100 ml.

Measurements of serum magnesium by atomic absorption spectrometry and by the colorimetric procedure of Bohuon[4] are compared in Figure 4. In analyses of twenty serums using the Beckman burners, the slope of the

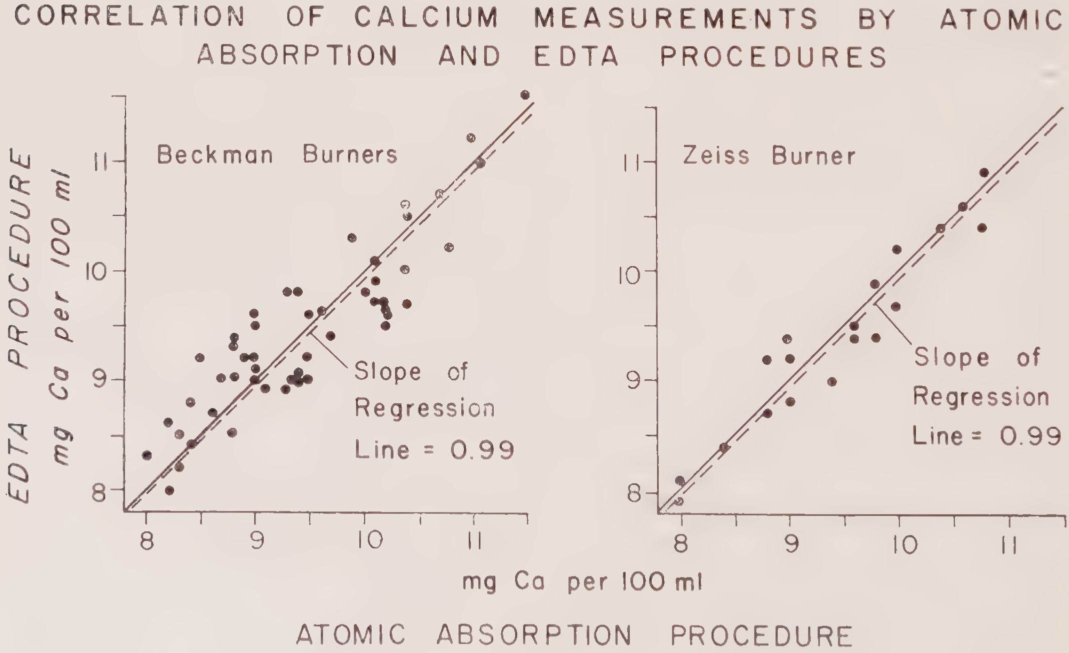

Fig. 3

regression line was 0.97, and the standard error of estimate was ± 0.29 mg. of magnesium per 100 ml. In analyses of twenty-four serums using the Zeiss burner, the slope of the regression line was 1.02, and the standard error of estimate was ± 0.09 mg. of magnesium per 100 ml.

Twenty ml. samples of serum were obtained from ten normal subjects. The concentrations of calcium and magnesium were determined in these samples by the Clark-Collip[6] and Briggs[5] procedures. With each of these samples, the means of replicate analyses of calcium and magnesium by atomic absorption spectrometry agreed with the means of replicate analyses by the reference procedures within ± 2 per cent.

SUMMARY

Atomic absorption spectrometry furnishes a rapid and sensitive method for the determination of calcium and magnesium in biological fluids.

Interferences in measurements of calcium due to sulfate and phosphate may be avoided by additions of strontium chloride to samples and standards. Interference of sodium in determinations of calcium may be compensated by additions of sodium chloride to the standard solutions. Interference of proteins in measurements of serum calcium and magnesium may be eliminated by precipitation of the proteins with trichloracetic acid.

If precautions are taken to avoid the various sources of interference and to minimize fluctuations in the burner system, atomic absorption spectrometry provides close correlations with determinations of serum calcium and magnesium by reference chemical procedures.

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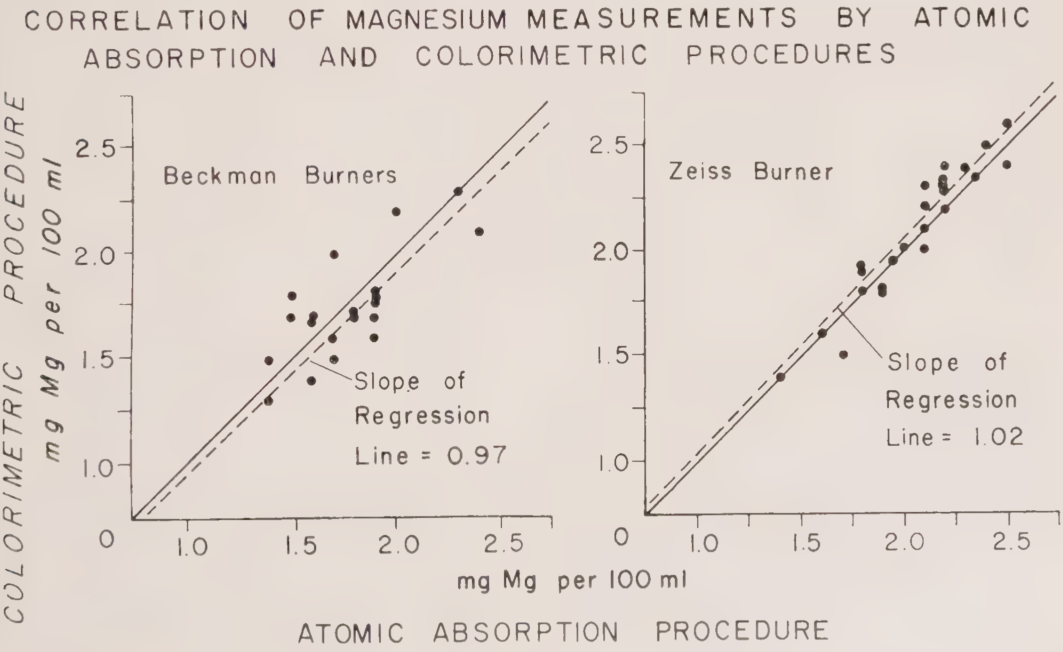

Fig. 4

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X-ray Spectroscopic Methods for Serum Electrolytes

SAMUEL NATELSON, Ph.D.

INTRODUCTION

The technic of x-ray spectrometry is based on the observation of Barkla[1] in 1909 and later Moseley[2], who demonstrated that the elements would emit energy in the x-ray region when irradiated with a beam of electrons or hard x-rays. The emission from each element was at a different wave length for corresponding lines, depending upon their atomic number. In the original work, photographic film was used. In this case, each element yielded sets of lines on the film located in a position characteristic of each element. These lines were subsequently identified with certain specific transitions from orbital to orbital.

The nearest orbital to the nucleus is called the K orbital; the others are called L, M, N, etc., in sequence. In this K orbital there are two electrons. The K lines are those which respond to the transition of an electron from an outer orbital to the K orbital. The L lines are emitted when electrons return from an outer orbital to the L orbital. Since the orbitals are composed of several orbits (3 for the L level and 5 for the M level), it is apparent that more than one K or L line will be obtained. Thus we have Kα, Kβ lines, Lα, Lβ, L etc., lines for each element. This gives rise to a series of lines characteristic of each element. By means of these lines the element can be identified, distinct from any other element. For this reason, x-ray spectrometric methods are specific. This is one main advantage over colorimetric methods. Since only a relatively few lines are obtained as compared to ordinary emission spectrometry (e.g., arc or flame photometry), background is low, and it becomes relatively simple to select a particular line for analysis.

Continuous development of instrumentation enabled the analysis of heavy metals to become practical about twenty years ago with commercially available instruments. Improvements consisted in developing x-ray tubes for excitation with targets of pure elements, the use of the gas flow detectors, Geiger counters, and scintillation counters for detecting emitted x-rays. Thus, a detector and counting equipment or a strip chart recorder replaced the use of photographic film. Suitable crystals were developed for diffracting the beam of x-rays so that lines could be isolated free of interference. For this same purpose, the pulse-height discriminator, now widely

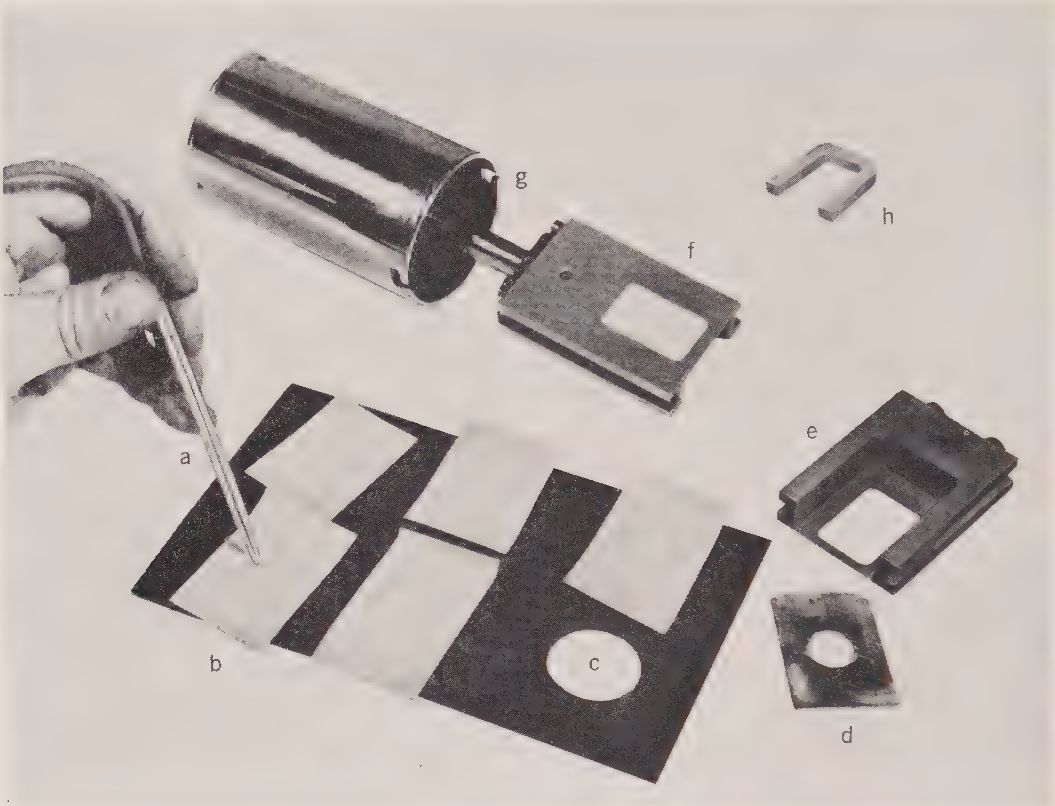

Fig. 1. A method for positioning the specimen accurately in the x-ray field. (a) is the pipet, (b) the paper with the continued spot, (c) the support to keep the paper suspended, (d) the mandrel around which the paper is folded and centered, and (e) is the cuvet holder. (f) is the cuvet ready for insertion into the field. (g) is both handle and x ray shield for inserting specimens into the field. The clip (h), is used to hold the paper in place while the aluminum mandrel (d) is slipped out.

applied in radioisotope counting, was also perfected. This is an electronic device which accepts and records signals from a detector only in a selected voltage range. Thus, if two lines are close to each other and cannot be completely resolved by the crystal, or if some of the original hard radiation reflects off the crystal, the pulse-height analyzer or discriminator will respond only to a preselected voltage range in the detector and reject a signal of higher or lower voltage.

With these improvements, analysis in the microgram range became practical only within the last ten years. In 1953, Castaing[3] first applied an electron microscope for the purpose of exciting the elements. This permitted the excitation of an area no larger than the field of an electron microscope. Thus, the elemental composition of a portion of a cell could be examined. Commercial instruments of this type are available and in use in many laboratories, particularly in examining dust particles and submicroscopic defects in alloys and minerals.

PRINCIPLE[4, 12, 16, 17]

A specimen of serum or blood of the order of 10 to 50 μl. is placed on a piece of filter paper with a circle (15 mm. i.d.) of polyethylene or tissue embedding wax. The serum is allowed to dry. The purpose of the circle of water repellant is to retain the sample in a confined area so that it distributes evenly and can be placed precisely in the same area of the x-ray field. The x-ray beam is then allowed to impinge in the specimen. Figure 1 illustrates this method of sample preparation.

The characteristic emission of the elements present (K lines for the lighter elements and L lines for the heavier elements) is then collimated by a series of parallel nickel plates. The collimated beam now impinges on a crystal, which acts to diffract the lines. The rays of the diffracted beam are allowed to pass through a second collimator, placed at the proper angle for the element sought. The x-ray beam now enters the detector where the number of counts observed is obtained on a scalar or ratemeter. The number of counts accumulated in a fixed time, or the time required to accumulate a fixed number of counts, is a function of the concentration of the element. This principle is illustrated in Figure 2.

By setting up a standard curve, one can then measure the concentration in the original sample, usually to ± 1 per cent. Thus, the instrument can readily distinguish between a serum calcium level of 10.0 and 10.1.

For the light elements (atomic number lower than for Titanium), a helium atmosphere or vacuum is required, since the x-ray emission for these elements is soft and absorbed by air. Only standard solutions are required for this procedure for the elements K, Ca, C1, total S, and total P in human serum and iron and zinc in whole blood.

It is apparent that the sample should be presented to the x-ray field in the form of an evenly-distributed, flat surface. Finely divided powders can be used, provided the size and composition of the particle of the matrix approximate that of the unknown. Solutions can also be used but these require some cover arrangement, such as with a thin film of Mylar or polyethylene, so as to prevent evaporation. Sections of tissue can be readily assayed for such elements as sulfur, phosphorus, or silicon.

The method of presenting the specimen evaporated to dryness, to the x-ray field, within a prescribed area is most

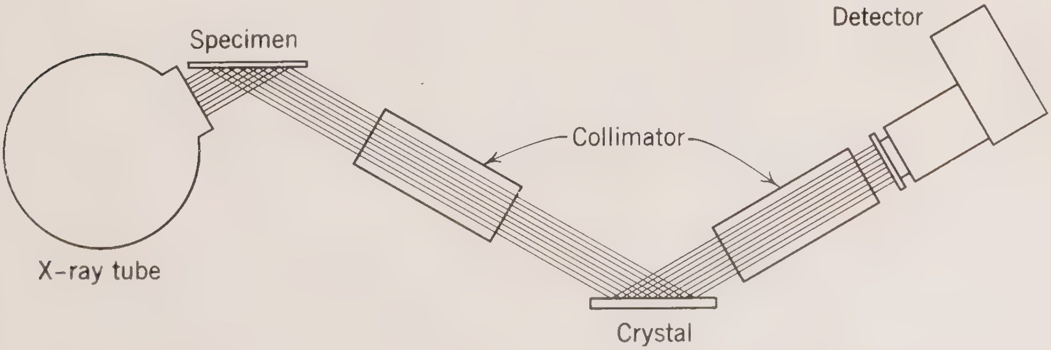

Fig. 2. Illustrating the principle of x-ray emission spectrometry.

practicable in clinical chemistry. Where the concentration is low, the sample can be evaporated to dryness in a confined area with the instrument of Figure 3.

Where the element may be volatilized and fixed on a flat carrier such as a paper disk, the instruments of Figures 4 and 5 may be employed. Figure 4 illustrates the capture on paper of arsenic as arsine with mercuric bromide. Phos-

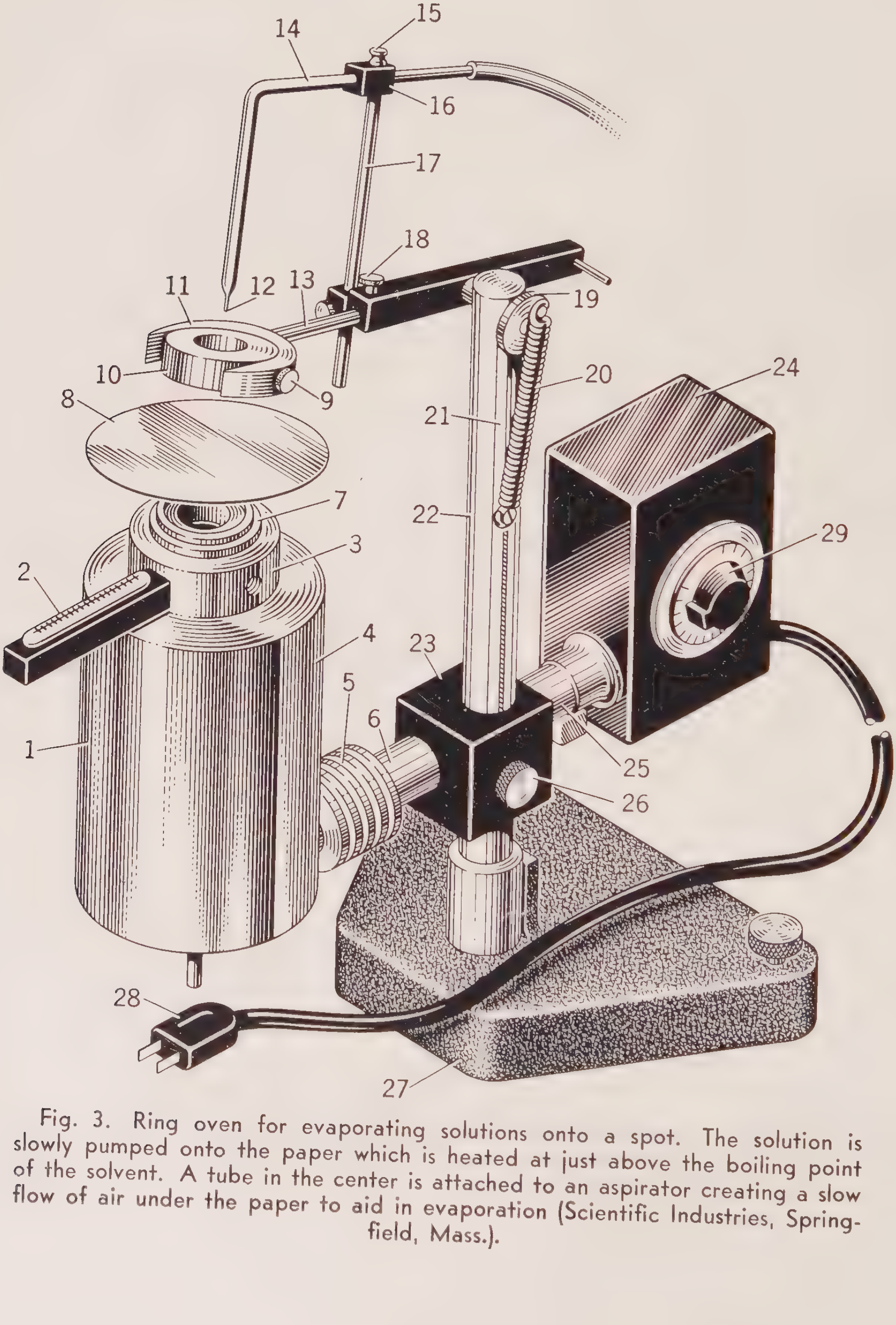

Fig. 3. Ring oven for evaporating solutions onto a spot. The solution is slowly pumped onto the paper which is heated at just above the boiling point of the solvent. A tube in the center is attached to an aspirator creating a slow flow of air under the paper to aid in evaporation (Scientific Industries, Springfield, Mass.).

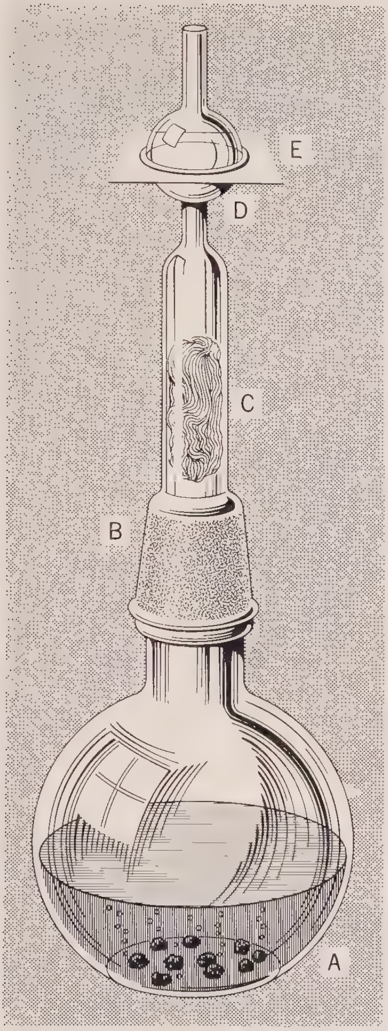

Fig. 4. Generator for distilling arsine and trapping on paper. (a) Zinc in acid solution generating hydrogen and arsine. (b) Glass ground joint. (c) Lead acetate-impregnated glass woll. (d) Ball joint. (e) Whatman No. 3 paper impregnated with mercuric bromide. Spring clamp for ball joint not shown.

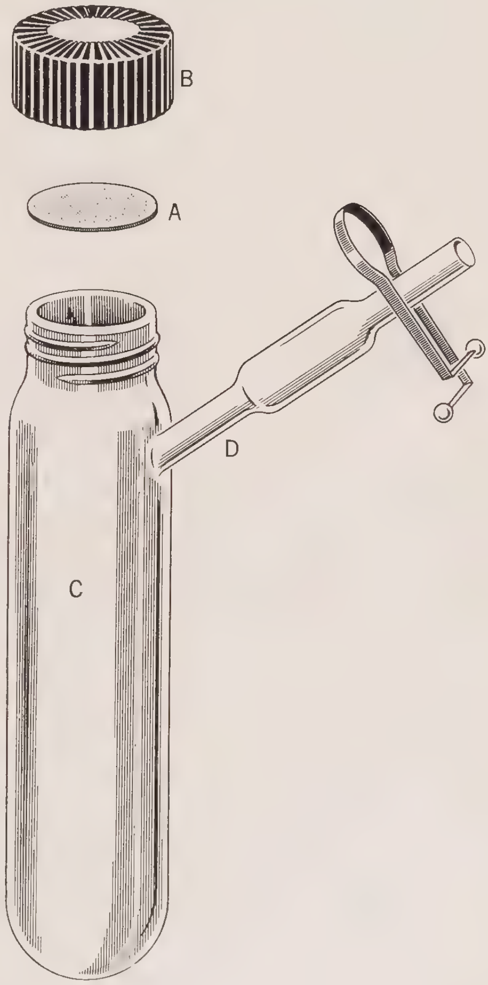

Fig. 5. Micro steam distillation apparatus. (a) is the paper with absorbant and (b) the crew cap with hole. (c) is made from a Pyrex screw cap tube (125 x 20 mm.) and contains the specimen. (d) is the side arm for adding the reagents.

phorus, as phosphine, can also be concentrated in this way from a large volume. Figure 5 illustrates an arrangement used by the author to digest serum protein and liberate protein-bound iodine by reagents added through the side arm. The iodine is fixed with alkailne phenol.

For elements such as mercury, lead, gold, bismuth, and some of the lighter elements such as zinc and copper, electroplating may be used to distribute the element on a metal disk or a plastic disk coated with graphite. An instrument used for this purpose is illustrated in Figure 6.

The instrument lends itself readily to automation. By placing specimens at regular intervals, in confined spots on paper, it is practicable to move the tape through the x-ray field. In this way, a tracing is obtained on the recorder, the

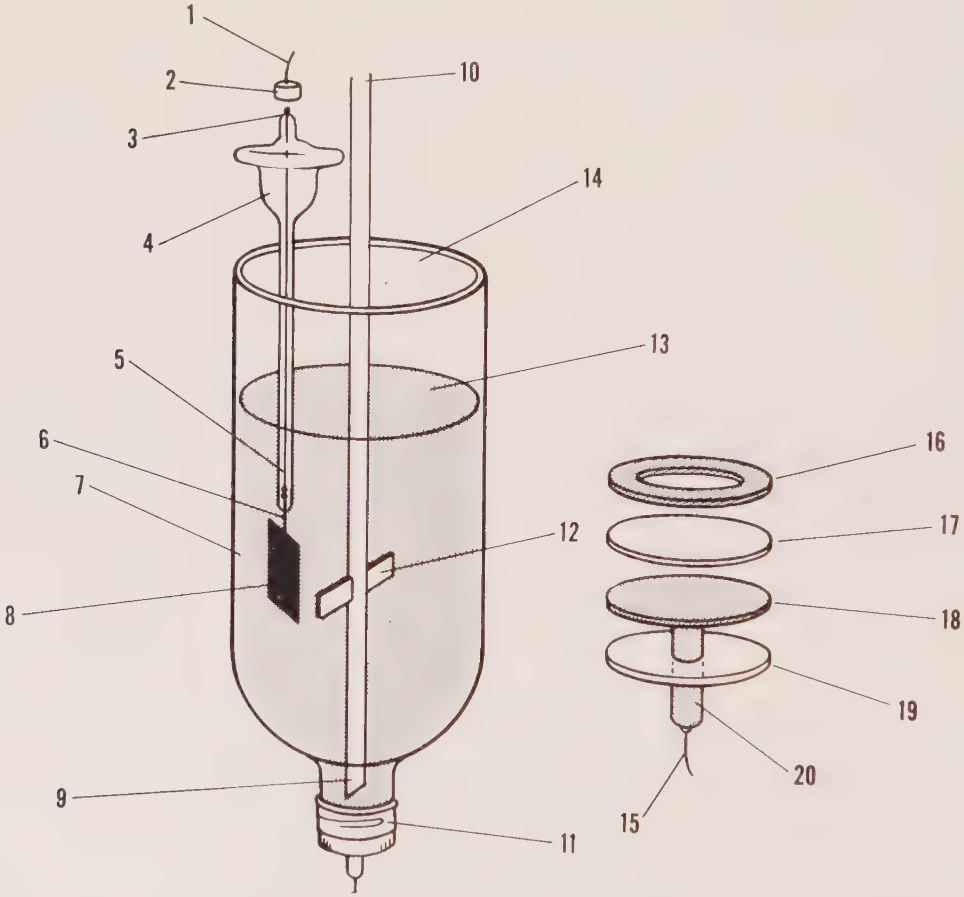

Fig. 6. Instrument for electroplating of certain elements. The plating takes place on the copper or graphite disk (17). Contact is made through copper disk (18). Gaskets (16) and (19) serve to insulate and confine the plating area. (1-7) and (8) comprise the platinum anode. (7) is the plastic container holding the plating solution. (9, 10) and (11) comprise the stirrer. The plating solution is (13). The screw cap has a hole bored into it to let the electrical contact (20) go through. The container is a polyethylene bottle with the bottom cut at (14).

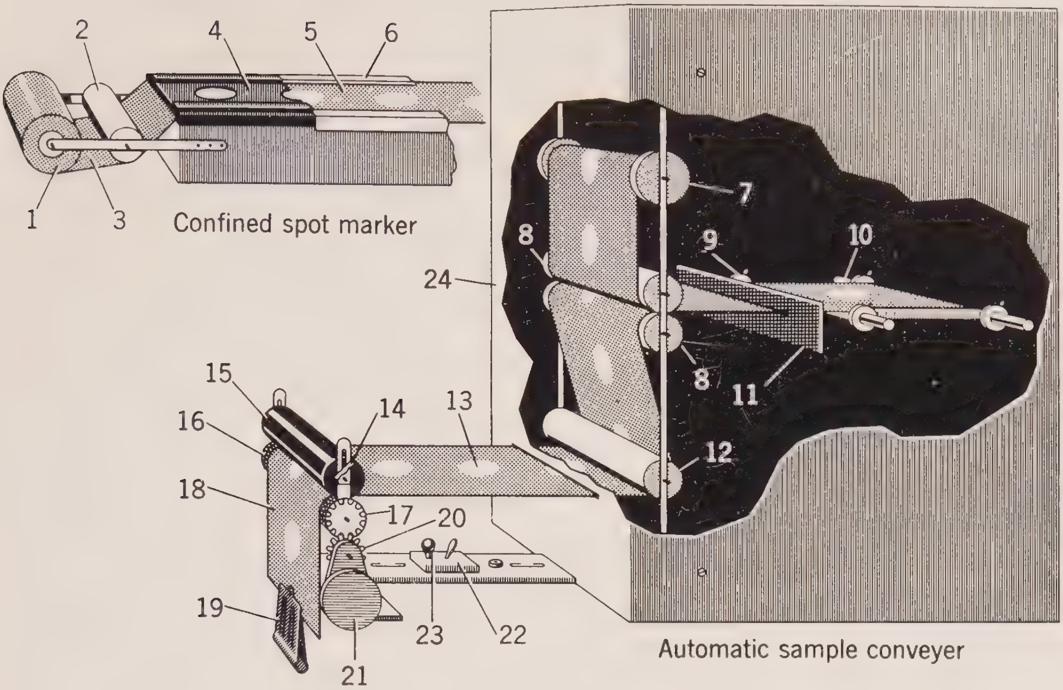

Fig. 7. Instrument for pulling paper strips through the x-ray field. The samples are measured into the confined spots and pulled through the x-ray field by the synchronous motor[21]. In the upper left-hand corner the instrument for preparing the wax circles is shown (see Clin. Chem. 6:300, 1960, for details).

peak heights of which are a measure of the concentration. Figure 7 shows an instrument designed by the author for this purpose.

An interesting application of this procedure is to scan electrophoresis strips for the sulfur content of the different protein fractions. Figures 8 and 9 illustrate this application. In this procedure, the strips are joined with cellophane tape. Since this tape contains sulfur, the change from one strip to the next is signalled by a peak which appears on the recorder. In figures 8 and 9 the protein content, as determined by staining and densitometry, is compared with the same strip scanned for its sulfur content before staining. Note that the protein content differs widely in its sulfur content in abnormal sera from different patients. Observe that in Figure 9, two serums which yield almost identical patterns by staining, vary widely in their sulfur content.

By the use of the slide changer, which moves specimens into the field and withdraws them into a magazine, it is practical to assay rapidly a specimen every twenty seconds. This has been applied to the determination of iron in blood as a measure of its hemoglobin concentration[8].

The x-ray spectrometer may be used for the determination of the presence of toxic elements by scanning at a fixed rate over a selected angle range. This permits the identification of the elements present and gives a fairly accu-

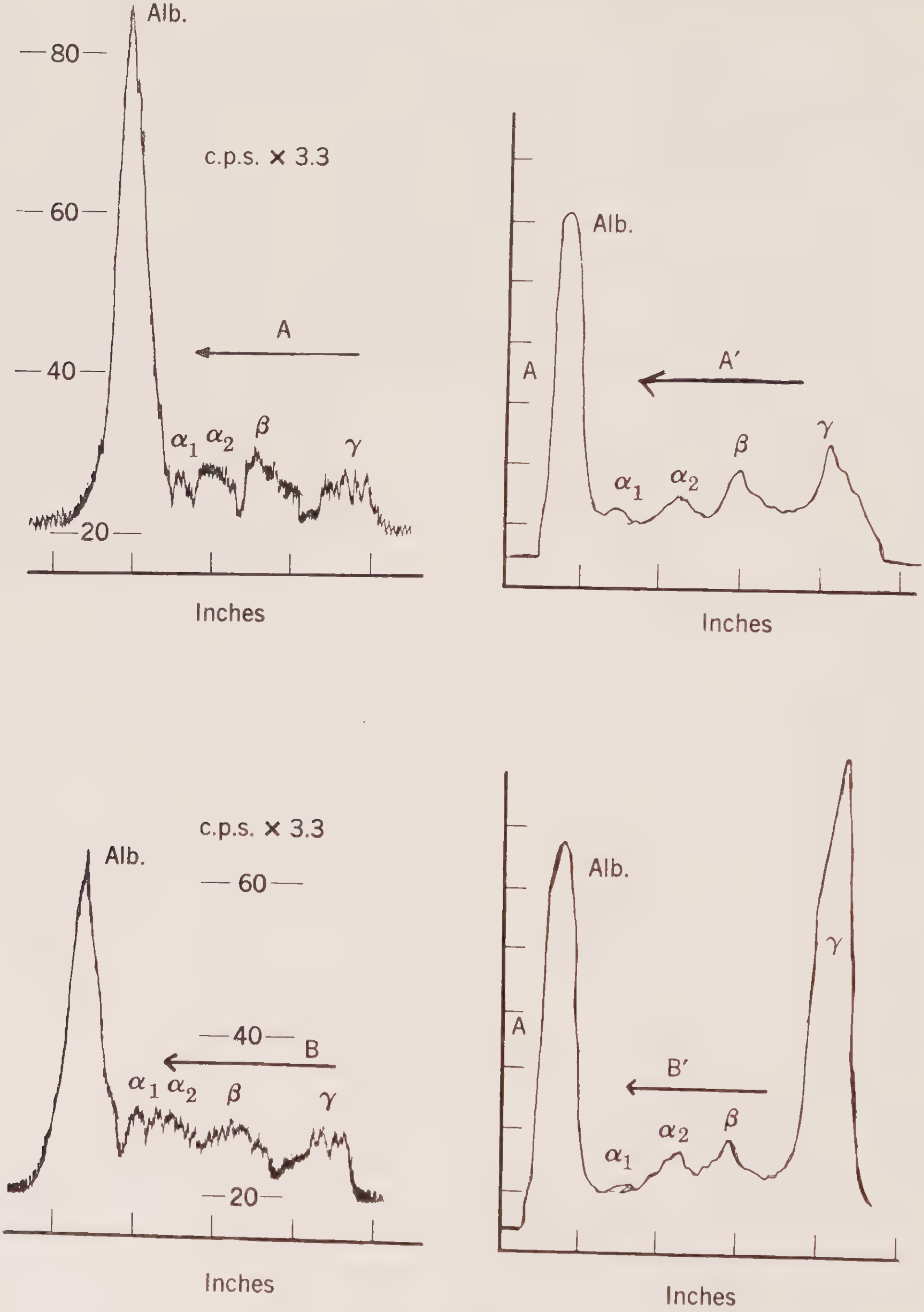

Fig. 8. Comparison of protein concentration in the serum protein fraction with sulfur content. Figures on the left are scanned for sulfur with the x-ray spectrometer. Figures on the right are the same pattern, then stained and scanned for protein. Comparison is made of a normal (above) with multiple myeloma (below). Note the low level of sulfur in the serum from the patient with multiple myeloma.

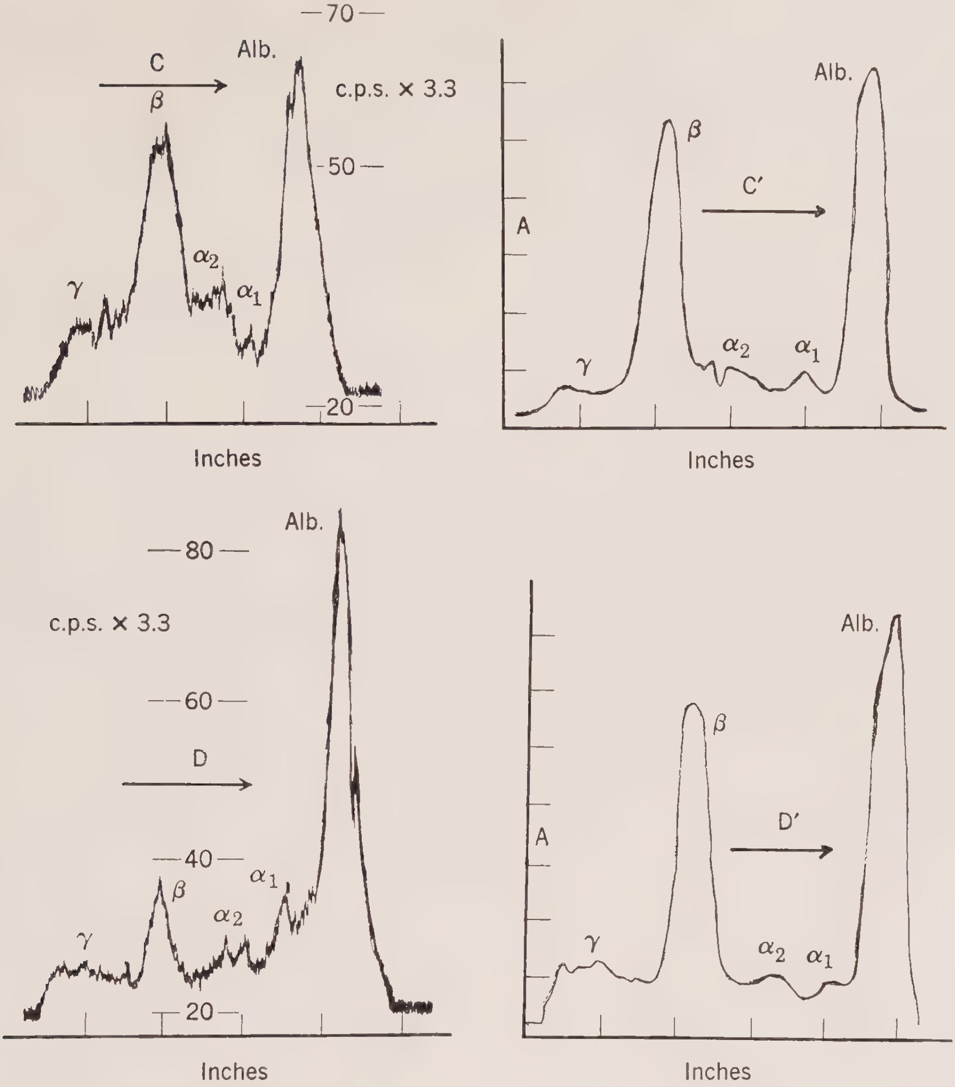

Fig. 9. Sera from two different patients with similar protein patterns obtained by electrophorasis and staining (right) as compared to the sulfur content of the fractions. Note that the patient above (multiple myeloma) has a high sulfur concentration in the beta fraction. Patient below has a low sulfur concentration in the beta fraction, (diagnosis not ascertained).

rate estimation of their concentration. For example, in scanning for the heavy elements in serum ash, both the Lα and Lβ lines may be observed on the same tracing. Since mercury and gold exhibit doublets in the Lβ region, one ascertains from this that these elements are actually present. There is a constant relationship between the peak heights of the Lα and Lβ lines, and this also serves to allow for specificity, difficult to obtain with other techniques. Figures 10 illustrates a typical tracing in this region.

The method of scanning may be

applied to the assay of the transition elements in human serum. Figure 11 illustrates this technique. It may be noted that copper is the most abundant, followed by zinc, iron, and nickel. Manganese, chromium and cobalt are not detected, since they occur in amounts of less than 3 μg per 100 ml. in serum.

If an electron microscope is used in lieu of the x-ray tube, particles smaller than a single cell can be assayed. The instrument, in this case, is called an electron probe. A schematic representation of one such instrument, commercially available, is illustrated in Figure 12.

REAGENTS

1. *Calcium.* The stock solution (100 mg. per 100 ml) is made up by dissolving 250 mg. of $CaCO_3$ in 20 ml. of water with 5 ml. of conc. HC1. The solution is now made to 100 ml. with water.

2. *Potassium.* The stock solution (100 mEq. per l.) is made by dissolving 7.456 gm. of KCl and making to 1 L. with water.

3. *Chlorine.* The stock solution (500 mEq. per l.) is made by dissolving 29.23 grams and making to 1 L. with water.

4. *Sulfur.* The stock solution (500 mM per l.) is made by dissolving 71.03 grams of anhydrous sodium sulfate and making to 1 L. with water.

5. *Phosphorus.* The stock solution (200 mg. P per 100 ml.) is made by dissolving 878 mg. of KH_2PO_4 in water and making to 100 ml.

6. *Iron (Whole Blood).* The stock

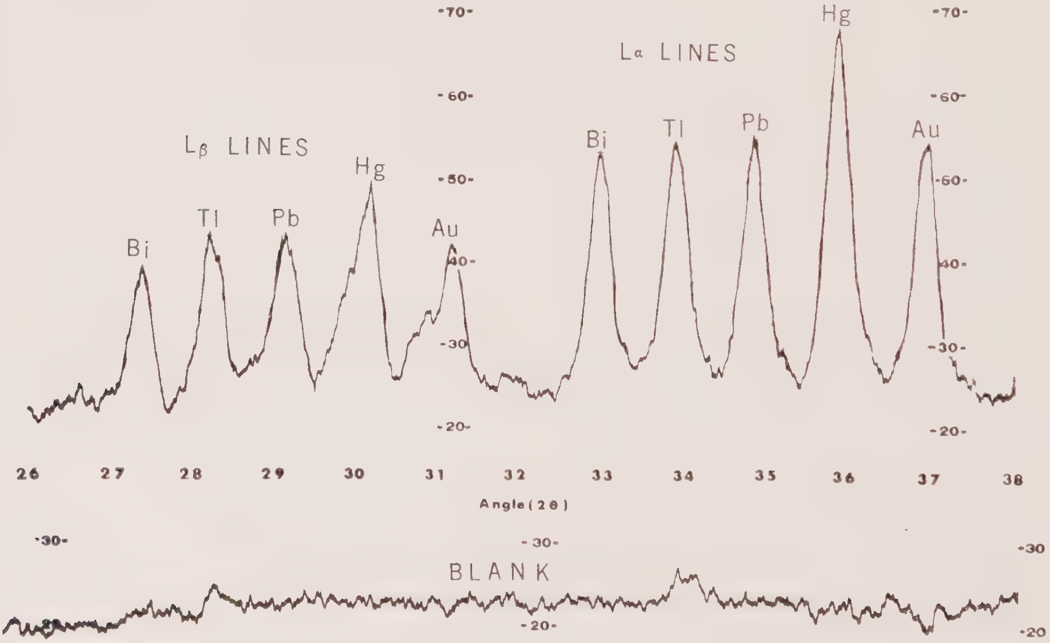

Fig. 10. Tracing for the heavy elements found in human serum in cases of intoxication. Scanning permits ready qualitative examination for these elements on one sample.

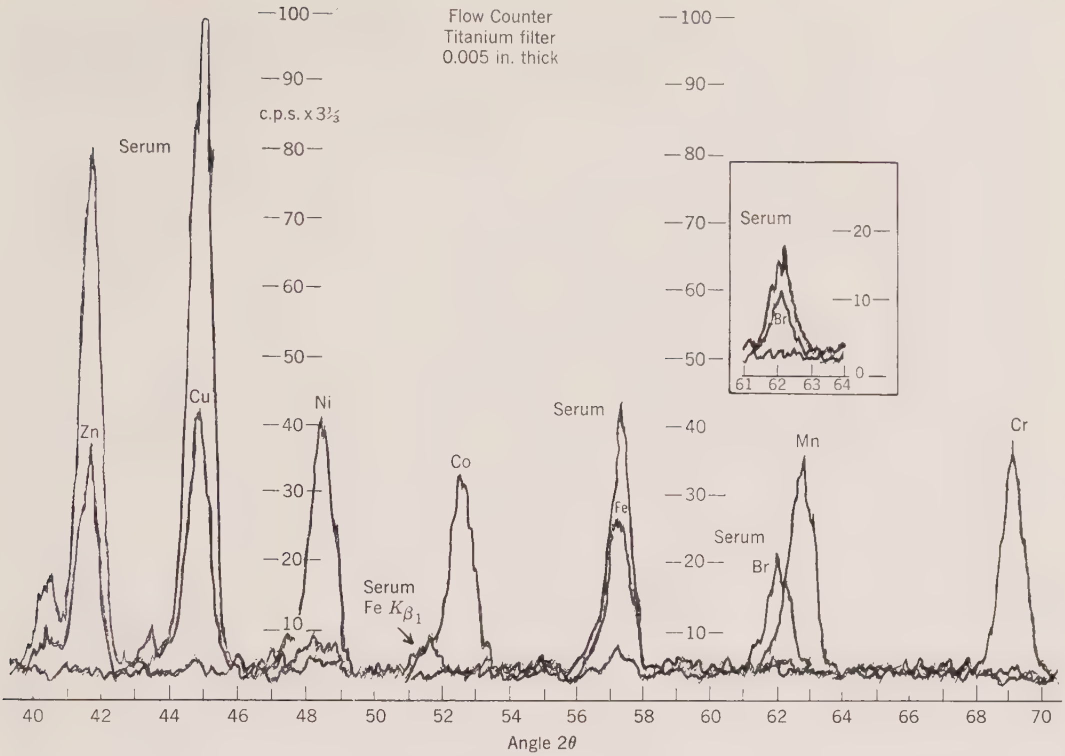

Fig. 11. Angle scan for the trace element in human serum using a 5-ml. titanium filter on the x-ray tube. Serum is compared to 2.5 μg. of standards except for Fe which is 1.9 μg., and a paper blank. Note the second order Kα line for bromine as proven by the inset.

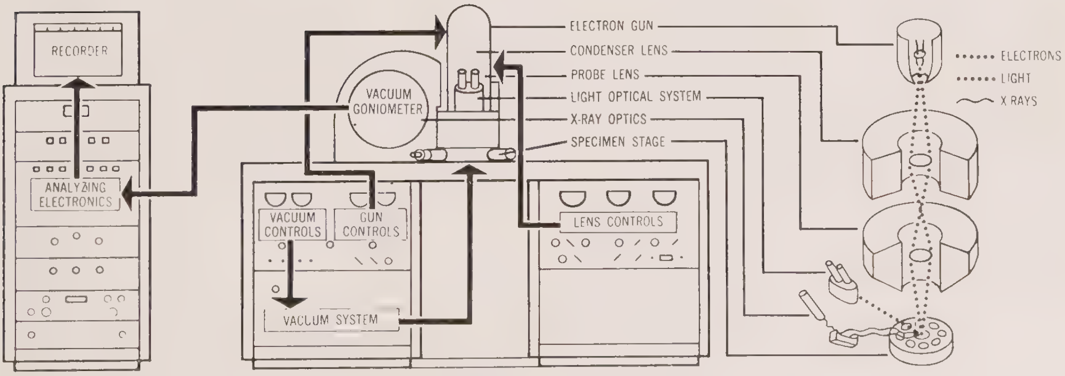

Fig. 12. Schematic drawing of the design of an electron probe (Philips Electronics). The electron beam excites the specimen, and the emitted x-ray beam is resolved for element identification and assay.

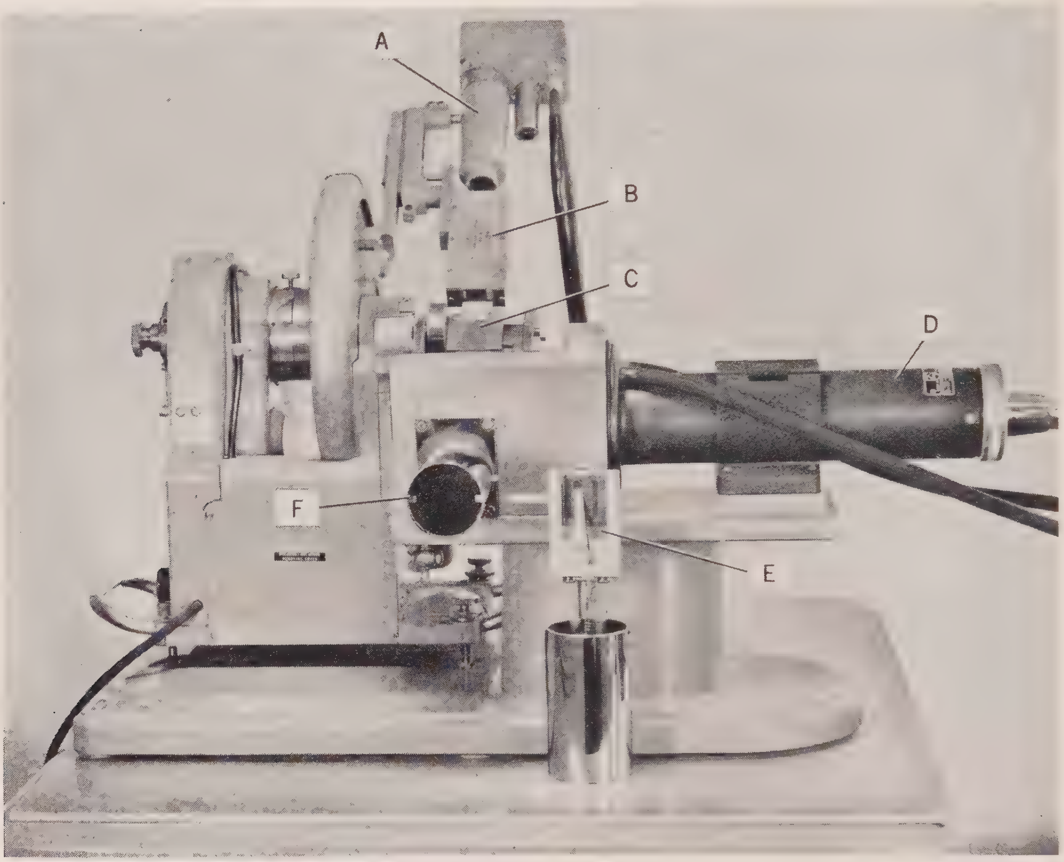

Fig. 13. Front view of the goniometer with the helium bag removed. (a) Detector. (b) Collimator. (c) Crystal. (d) X-ray tube. (e) Rear of sample holder with a clip holding a flat specimen in place. This is inserted into (f) the metal guides aligning the specimen holder. Specimen lies horizontally while the detector moves in a vertical plane. (Philips Electronics.)

solution (200 mg. Fe per 100 ml.) is made by dissolving 1.404 grams of ferrous ammonium sulfate, $(NH_4)_2SO_4 \cdot FeSO_4 \cdot 6H_2O$ in 30 ml. of 1N hydrochloric acid and diluting to 100 ml. with water.

7. *Zinc (Whole Blood).* The stock solution (100 mg. Zn per 100 ml.) is made by suspending 124 mg. of ZnO in 10 ml. of water and adding 3 ml. of conc. HC1. The solution is made to 100 ml. with water.

Dilute Standards. The stock standards listed above are diluted to encompass the range expected to be found in human blood or serum.

APPARATUS

1. *Whatman #40 Paper.* This paper can be supplied in rolls 1¼ in. wide. Three-inch lengths are cut off, and a circle of wax (Bioloid wax) is made by melting the wax, dipping a warmed cork-borer (#12) into the warmed wax, and stamping the circles in the center of the paper. Allied Chemical, low-mol.-wt. polyethylene can be used for the same purpose.

2. *X-ray Spectrograph.* X-ray spectrographic equipment is available in the United States from the Philips Electronics Company, the General Electric Company, the Picker X-ray Company, and the Siemens America Company. All available equipment uses simliar instrumentation. An x-ray tube is powered by a regulated power supply which is fed into a high voltage transformer (50-100 K.V.). The output of the transformer operates the x-ray tube, which is housed in a casting so that the beam of x-rays can impinge on the sample. This section of the instrument is called the spectrometer. It also includes room for a collimator, a crystal holder, and a support for a detector. Figure 13 illustrates an assembly showing the standard spectrograph of Philips Electronics Co. In this case, the helium bag has been removed in order to show the location of the crystal and other component parts.

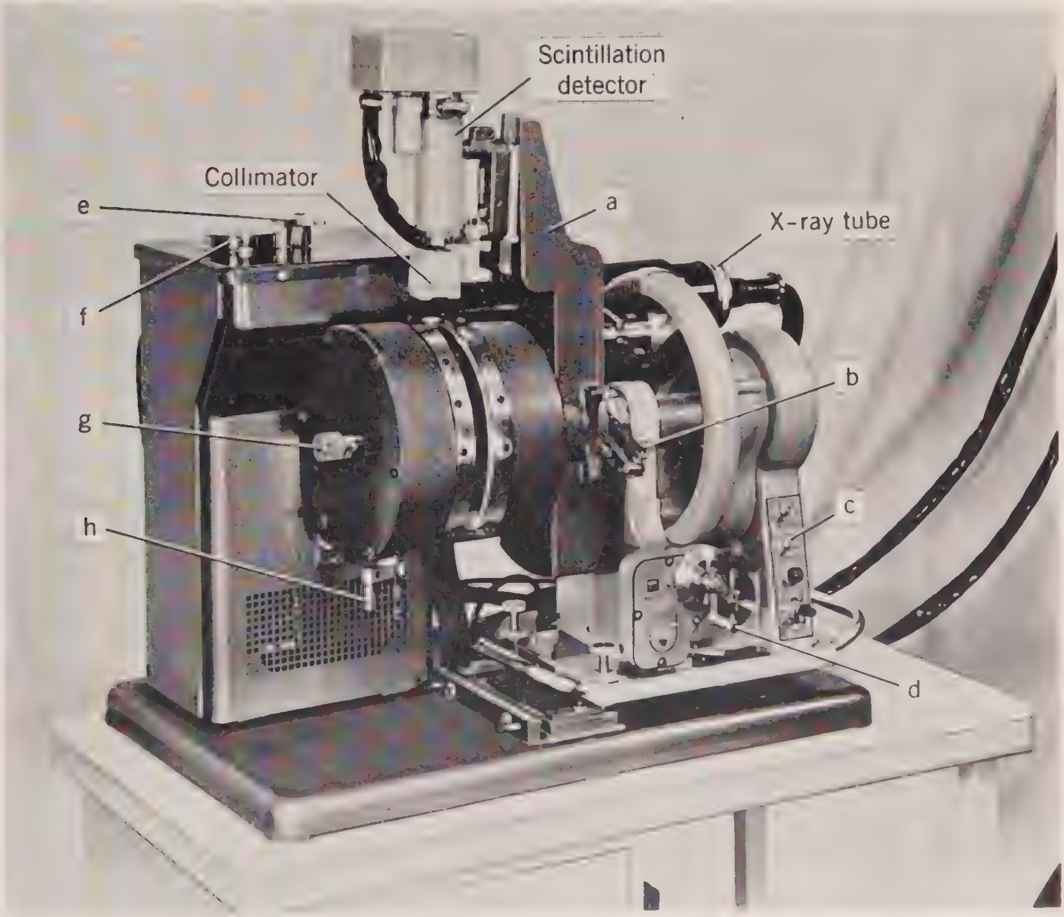

Fig. 14. The vacuum spectrograph. (a) Movable arm supporting the detector. (b) Adjusting screw to rotate the crystal. Switch (c) turns on the motor for automatic angle scanning. (d) Crank for manual scanning. (e) Permits rotation of the sample-containing block. (f) Removable cover, to add or remove the samples, fitted with a rubber gasket to hold vacuum. (g) Vacuum release valve. (h) is the arm which is moved to change the two crystals contained in the instrument. (Philips Electronics.)

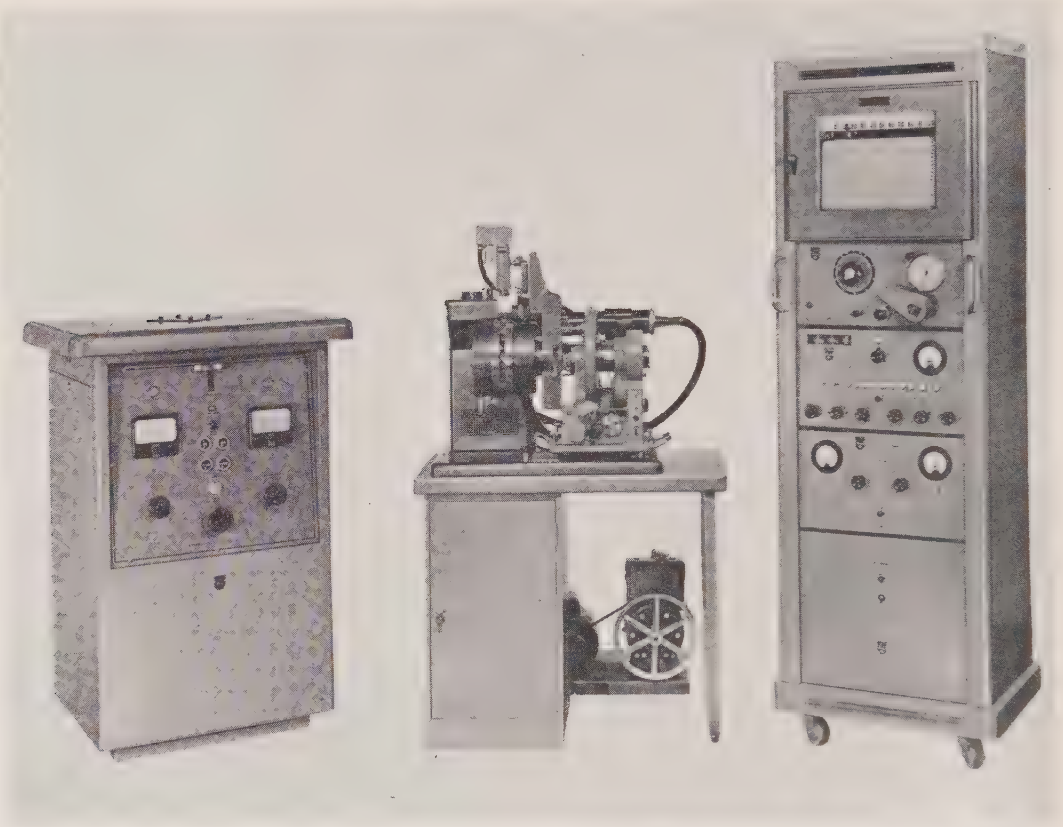

Fig. 15. General view of the Philips vacuum x-ray spectrometer assembly. The high-voltage transformer and controls are housed in the panel on the extreme left. The recorder and circuit panel (stack) are on the extreme right. The vacuum spectrograph sits on a separate table in the center with the vacuum pump below.

Figure 14 demonstrates the vacuum spectrograph. For the lighter elements, the casing permits evacuation. Significant improvement with the vacuum, as compared to helium, is only observed with elements of atomic number of the order of magnesium, sodium, or less.

In the vacuum spectrograph, a sample changer permits the analysis of ten specimens before the vacuum need be broken. The instrument may be set so that a reading is taken for a fixed number of counts or a fixed time, then rotated mechanically to the next position. Thus, the instrument may go unattended while the results are reported on a digital readout.

The crystal holder and detector are revolved to a desired angle by the goniometer. The goniometer is an angle-measuring device and a means to change the angle. The detector output is fed to an amplifier and finally to a rate meter and scalar for taking a fixed number of counts, counting for a fixed time or recording the counts on a recorder. The recorder, rate meter, scalar and primary power supply are stacked vertically so as to form the panel.

The Philips instrument is illustrated

in Figure 15. The housing on the left contains the high voltage transformer. On the right is the panel. Supported on the table in the center is the vacuum spectrograph.

In summary then, the instrument comprises a spectrometer, a goniometer, an x-ray tube, a detector, and a panel. These units are supplied in all commercial equipment.

PROCEDURES

Sample Preparation. On Whatman No. 40 paper, 1¼ in. wide, into the center of the wax circles is measured 25 μl. of serum for Ca, K or C1. For sulfur or phosphorus, 50 μl. of serum is used. For total iron, 0.1 ml. of water is added to 0.1 ml. of heparinized blood to hemolyze the red cells. The solution is centrifuged, and 50 μl. of the sample are measured into the confined spot with a micropipet. The samples are allowed to air dry. They are now ready for assay.

Preparing the Instrument. A drop of the stock solution is placed in the confined spot and allowed to dry. This solution is used to line up the instrument and also to select a pulse height discriminator range for the particular element. Once the instrument is lined up for a particular element, it is in line for the other elements. Only the pulse height range needs to be changed. A tungsten target x-ray tube is suitable for the elements listed. For all the elements listed under reagents, other than iron, the chromium target tube is preferred for higher sensitivity.

The paper is folded over an aluminum plate (mandrel) to center the spot in line with a hole in the aluminum plate. The aluminum plate is now inserted into the cuvet holder. A plastic clip is inserted to hold the paper in place, and the aluminum plate is slipped out. The cuvet is now inserted in the x-ray field. A suitable crystal is inserted in the crystal holder in the goniometer; lithium fluoride for potassium, iron, and calcium; sodium chloride for sulfur and chlorine; or quartz for phosphorus. Helium flow and p-10 gas flow are now started.

The pulse height discriminator is opened wide to allow all emission to be detected. The goniometer is turned to the angle for the element selected. The crystal is now adjusted for maximum response on the rate meter. The angle is changed by ± 0.1 degree intervals, and the crystal is adjusted each time for maximum response. This is repeated until the maximum response is observed. Thus, a best position for the crystal and angle is sought. This is called "rocking in the crystal." This need be done only once for the instrument. For routine analysis, this position should be checked only if a sudden decrease in sensitivity is observed. The p-10 gas flow counter is now adjusted to obtain maximum response on the rate meter. The collimator adjacent to the detector is also adjusted for maximum response. The instrument is now lined up and ready for use.

Obtaining the Pulse Height Discriminator Range. This is done by setting a narrow window (0.5 volts) on the band-width dial and scanning with the baseline dial. This will draw a curve on the recorder indicating the voltage range to be selected for the individual element. By inspection of the curve, one arrives at a setting for the baseline dial and the band-width dial for the particular element. This voltage range will vary from instrument to instrument, and is dependent on the voltage on the counter (detector). Voltage on the detector usually ranges from 1350 to 1700 volts, depending upon the instrument used. In general, it is best to adjust the voltage on the detector to as low a value as possible, without having the pulse height analyzer curve running into background. The pulse height analyzer is made with an evaporated drop of the stock solution in the x-ray field as described for lining up the instrument.

Method of Assay. A standard curve is made by using the confined spot paper and adding to it the volume previously indicated. The same pipet is used for standards and unknowns. The dried samples are placed in the field, and the instrument is set to record 10,000 counts. The time required to accumulate that number of counts is a measure of the concentration. The sample is usually counted for three times and averaged. For this purpose the rate meter is turned off, and the scalar is used with the dial set for "fixed count." A press of a button or lever starts the timer. It is reset when the reset button is pushed.

Calculations:

$$\frac{10{,}000}{\text{time (seconds)}} = \text{counts per second (C.P.S.)}$$

$$\text{(C.P.S.) unknown} - \text{(C.P.S.) blank} = \text{(C.P.S.) net}$$

A plot is made of the net C.P.S. for the standards, and the concentrations of unknowns are read from the curve. The standards used depend on the range expected from normal and abnormal serum. For example, for calcium, one would normally use the 5, 10, and 15 mg. per 100 ml. standards for the standard curve. Commercial serum controls should be used to check the procedure. Usually the standard curve intercepts the origin. In this case, one can use the following calculations:

$$\frac{\text{net C.P.S. unknown}}{\text{net C.P.S. standard}} \times \text{concentration of Std.} = \text{concentration of unknown}$$

DISCUSSION

The main advantages of x-ray spectrometry are its specificity and non-destructive nature so that the same sample can be assayed again and again. For the elements discussed, no reagents are required. A small sample is needed, and the technic is simple, once the instrument is set up. In effect, the procedure can be described as placing a sample on a piece of paper, allowing it to dry, and inserting it into an instrument. After pressing a button, the results are available in a short time. The instrument lends itself readily to automation and simultaneous assay for several elements.

The major unsolved problem for the clinical chemist is the assay of sodium. If this could be accomplished, a simultaneous automated instrument for the electrolytes (including chlorine) is readily available. The problem arises from the fact that sodium emission is so soft that it is stopped by even thin layers of *Mylar* on the counter. Recently, a polypropylene window has been developed which meets this problem[15]. However, self-absorption in the sample still limits its sensitivity. Present instrumentation is available which can solve this problem, but it is only available on an experimental basis. This newer instrumentation comprises the use of aluminum target tubes, thinner windows on the x-ray tube, and x-ray tubes with higher currents. The sample, in conventional instrumentation, is about ten inches from the detector; closer coupling should improve sensitivity.

Other elements, such as strontium[9], bromine[10], Fe in serum, PBI[11], heavy metals[14], and trace metals[18], can be assayed only after they are concentrated. Only if this instrument became practicable for the electrolytes would its use be extended to these problems, because of its high initial cost.

SOURCES OF ERROR

The inherent sources of error in this procedure are the errors in pipetting and counting. Errors due to placing the specimen improperly in the field are also possible but can be avoided with a reasonable amount of care.

For one standard deviation, the error in counting is calculated as the inverse of the square root of the number of counts taken. For example, if one accumulates 30,000 counts, as recommended in the procedure, then the error will be one part in 174 for one standard deviation, two parts in 174, or approximately 1 per cent error for two standard deviations. Thus, the counting error is not the source of major difficulty. The major source of error is in the pipetting. Pipetting of samples of the order of 25 to 50 μl. can be done with a reproducibility (1 std. dev.) of ± 0.5 per cent, if the Richard Allen Co. (Evanston, Ill.) pipets with bulbs are used, as determined in our laboratory. Thus, pipetting error should not exceed ± 1 per cent. In combining these two errors, one takes the square root of the sum of the squares of the errors. Thus, assuming a 1 per cent error in counting and a 1 per cent error in pipetting, one obtains an error of ± 1.4 per cent as a total error (2 std. dev.). This can readily be achieved in actual practice.

RANGES OF VALUES

The range of values of the various elements is listed in Table I. The range is determined as two standard deviations from the mean.

The normal range for iron is, in effect, a function of the total hemoglobin range. Serum iron contributes very little to this determination, since the mean of the normal is only 76 μg. per 100 ml., as determined by x-ray spectrometry with a range of ± 7.5 μg.[8].

For zinc, the total content in whole blood is a function of the carbonic anhydrase content of the red cells and is, therefore, a function of the hematocrit. Accordingly, zinc assay is done on packed cells or plasma or serum. The concentration of serum zinc is of the order of 120 μg. per 100 ml., and it accounts for about 10 per cent of the zinc content of whole blood[13].

Total phosphorus is essentially a sum of lipid phosphorus and inorganic phosphorus[8]. By subtracting inorganic phosphorus, lipid phosphorus is obtained. Lipid phosphorus (mg. per 100 ml.) multiplied by 25 yields the phospholipids in serum.

The sulfur in serum is concentrated

TABLE I "NORMAL" RANGE OF VALUES (2 S.D.) AS DETERMINED ON THE X-RAY SPECTROMETER

ELEMENT	SAMPLE	MEAN	RANGE (2 s)
Potassium	serum	4.6 mEq./L.	± 0.25
Chlorine	serum	102 mEq./L.	± 2.0
Calcium	serum	9.9 mg./100 ml.	± 0.3
Iron	whole blood (male)	50.7 mg./100 ml.	± 10.1
	whole blood (female)	44.9 mg./100 ml.	± 8.9
Zinc	packed red cells	1,406 μg/100 ml.	± 200
Total Phosphorus	serum	13.3 mg./100 ml.	± 1.0
Total Sulfur	serum	29.2 mM/L.	± 4.0

in the protein fractions. The protein-free filtrates of serum contain approximately 1 mM only of sulfur per L. Thus, the total sulfur level is normally directly related to the total protein level[6]. Abnormal protein in serum often contain very little sulfur. For this reason, total sulfur cannot be used as a practical method for determining total protein[7].

RÉSUMÉ OF CLINICAL INTERPRETATIONS

The clinical interpretation for variation in serum calcium, potassium, and chlorine levels will not be discussed since they are adequately treated in other chapters.

Increased concentrations of zinc in blood have been found in anemias and respiratory diseases. Low zinc values have been observed as a result of a nutritional deficiency endemic to Egypt.

Total phosphorus levels are elevated in the lipemias, and they run roughly parallel with elevations of the cholesterol level. While the inorganic phosphorus level is usually of the order of one-third to one-fourth of the total phosphorus level in the normal, this ratio will drop to as low as one-twentieth in lipemias such as lipoid nephrosis. When, in a lipemia, the fat separates spontaneously and floats to the top, it is generally indicative of a lowered ratio of phospholipid to total lipid level. In these cases the cholesterol/phospholipid ratio will also be high.

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The Role of the Adrenals in the Metabolism of Sodium and Potassium

JAMES E. CLARK, M.D.

Sodium and potassium metabolism is now known to be primarily regulated by the mineralocorticoid, *aldosterone,* produced by the glomerulosa layer of the adrenal cortex[1]. The recognition of this has been an interestingly woven story which began with an amorphous residue highly active by bioassay producing urinary retention of sodium and excretion of potassium. Kendall's "*amorphous fraction*" became the object of considerable research, resulting eventually in the isolation and chemical elucidation[2] of a new steroid produced by the adrenal cortex, which was named *aldosterone* because of the position of the aldehyde group at carbon 18[3]. The adrenal effect on sodium and potassium regulation is not entirely confined to *aldosterone,* however, although this mineralocorticoid is the most potent hormone concerned with these electrolytes.

For a thorough appreciation of the role of the adrenals, we must consider both the adrenal cortex and the adrenal medulla. Whereas *ACTH* liberated by the anterior lobe of the pituitary is of primary importance in glucocorticoid release from the adrenal cortex, it probably plays a relatively minor role (if an important one at all) in the release of *aldosterone*[4]. Although many theories have been advanced in the past concerning the regulation of the adrenal release of *aldosterone*, the current concept[5–10] evokes the juxtaglomerular apparatus of the afferent arteriole of the kidney in a chain reaction, liberating renin with the eventual production of angiotensin I and angiotensin II. Both renin and angiotensin II have been shown to stimulate *aldosterone* secretion from the adrenal cortex which, in turn, exerts its major effect on the renal tubule. In considering the action of the mineralocorticoid, *aldosterone*, on the kidney, it should be noted that *aldosterone* exerts no significant effect upon the glomerular filtration rate or on the effective renal plasma flow[11]. It was concluded earlier[12–14] that *aldosterone* acts predominantly on "distal" regions of the nephron. More recently[11], it has been shown that *aldosterone* enhances directly sodium and chloride reabsorption in the *pars convuluta* of the distal tubule, and also augments sodium and potassium exchange in the late distal tubule and in the collecting duct. The glucocorticoids, hydrocortisone and cortisone, increase glomerular filtration rate and effective renal plasma flow, enhance sodium transport in the *pars recta* of the distal tubule, and augment sodium and potassium exchange in the late distal tubule and in the collecting duct[11].

It should be emphasized that the mineralocorticoid, *aldosterone,* plays by far the most important role in the reabsorption of sodium and the excretion of potassium. The effect of glucocorticoid on sodium transport and sodium-potassium exchange[15] is quite minimal in comparison, and is rarely important as a clinical problem.

The principle effect of the medullary amines, epinephrine and norepinephrine, is on the renal circulation with a decrease in effective renal plasma flow and glomerular filtration rate[15]. The medullary amines depress potassium excretion acutely and chronically[16–18], although the mechanism of this depression is not fully understood. They also decrease the urine acidity[19] which is paradoxical, considering that there is usually an inverse relationship between urinary potassium and acid excretion rates. The mechanisms for these changes are at present unknown.

Of the clinical syndromes which result in abnormal sodium and potassium metabolism, one of the earliest which was well described was primary hyperaldosteronism or Conn's syndrome[20]. In this syndrome a tumor[21] of the adrenal cortex or diffuse hyperplasia[22] of the adrenal cortex of both adrenal glands is the initiating factor in an autonomous secretion of *aldosterone.* This affects the renal tubule to evoke sodium retention and marked potassium wasting[23]. This results in a clinical syndrome[24] characterized by intermittent tetany, paresthesia, periodic severe weakness and paralysis, polyuria and polydipsia, arterial hypertension, and in most cases, absence of edema. The biochemical manifestations of Conn's syndrome are those of an hypokalemic alkalosis usually with an elevated serum sodium. The potassium concentration in sweat, saliva, and urine is high, whereas the concentration of sodium in sweat, saliva, and urine is low[25]. Bioassay of urinary extracts shows high sodium-retaining activity, and *aldosterone* excretion and secretion studies show elevated values.

A most important feature observed in primary hyperaldosteronism is that the clinical manifestations of the syndrome usually occur while the patient is on a diet normal in sodium content[26]. If a patient with this syndrome is being treated with a diet which is very low in sodium content, presumably not enough filtered sodium will reach the distal portions of the tubule to be available for exchange with potassium, thereby decreasing the amount of potassium lost into the tubular urine and hence from the body. If potassium loss does not occur, then the symptoms of tetany, paresthesias, weakness, and paralysis do not follow. Furthermore, the polyuria and polydipsia, which are secondary to the so-called kaliopenic nephropathy[27–29], (thereby rendering the tubules refractory to antidiuretic hormone) likewise do not occur. Therefore, under the circumstances of a reduced sodium intake, none of the clinical features will be present, nor will the biochemical abnormality of an hypokalemic alkalosis be evident. If, on the other hand, an attempt is made to correct the hypokalemia by potassium replacement, this is usually unsuccessful, for the only way to abolish the potassium loss is by witholding sodium from the patient. The treatment of this syndrome is to remove the adenoma or to perform a bilateral adrenalectomy.

So-called secondary hyperaldosteronism, which is seen in patients with cirrhosis with ascites[30]; the nephrotic syndrome[31]; pregnant diabetics; patients who have either a low sodium diet, a high potassium diet, or a low effective blood volume or dehydration, all appear to be manifest by the same mechanism[32]. These stimuli affect the juxtaglomerular apparatus to become hyperplastic with an increase in the number of granules[5]. These granules presumably provide an increased source of renin release, thereby making available more angiotensin I and angiotensin II, and either through renin, angiotensin II, or both, *aldosterone* is secreted to affect the renal tubule in exchange of potassium for sodium with a result in sodium retention and potassium loss. Edema is a common problem, and hypertension is rare in contrast to primary hyperaldosteronism. Another clinical entity involving *aldosterone* is that of selective hypoaldosteronism[33–34]. In this syndrome, there appears to be a normal regulation of the glucocorticoids from the adrenal cortex as evidenced by normal urinary and plasma glucocorticoids in response to *ACTH* stimulation. There is a decrease in *aldosterone* secretion resulting in potassium retention and sodium-wasting by the kidney. The clinical aspects of this syndrome may be due to the potassium retention, as evidenced by hyperkalemia associated with electrocardiographic changes of widening of the QRS complex to bundle branch block proportions, or of prolongation of the PR interval until heart block with occasional Stokes-Adams syncope occurs; or the syndrome can be manifest primarily by sodium-wasting, producing symptoms associated with hyponatremia. The etiology of this is not known, and no studies have yet been published in which the juxtaglomerular apparatus has been carefully observed in this syndrome.

Within the past several years, Bartter[35] has described another syndrome associated with juxtaglomerular hyperplasia, in which the juxtaglomerular apparatus is enlarged with increased numbers of large granules. This is associated, presumably, with an increased production of renin with resulting increases in angiotensin I and II stimulating the adrenal cortex to produce *aldosterone* in large amounts, thus acting on the renal tubules to produce potassium wasting and sodium retention with a resultant hypokalemic alkalosis. The blood pressure is normal or low in these patients. It has been felt that a possible explanation for this is that there is a refractoriness of the peripheral arterioles to angiotensin II. Several patients with this syndrome have had high levels of angiotensin II, and have failed to respond to exogenous doses of angiotensin II, which would otherwise produce a rise in blood pressure in a normal individual.

In conclusion, it appears that sodium and potassium metabolism is primarily regulated by the mineralocorticoid of the adrenal cortex, *aldosterone*. *Aldosterone* directly enhances sodium reabsorption in the distal convoluted tubule and augments sodium and potassium exchange in the late distal tubule and the collecting duct. Although glucocorticoids manifest sodium-retaining and potassium-losing properties, these effects may be masked by their stimulating effect on glomerular filtration rate. The adrenal medullary amines act primarily to depress the effective renal plasma flow, and in addition depress potassium excretion and urinary acidity.

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The Role of Sodium in the Transport of Non-electrolytes

IRWIN H. ROSENBERG, M.D. and LEON E. ROSENBERG, M.D.

The participation of sodium ion in the transport of organic non-electrolytes was first demonstrated, in 1958, by Riklis and Quastel, in the course of studies of glucose transport by isolated guinea pig intestine[1]. Since this report, the influence of the sodium ion in numerous transport systems has been studied[2–13]. In a host of preparations, this ion has been shown to be unique among cations in its ability to stimulate active transport. The list of substances whose transport is dependent on the presence of sodium ion in the extracellular environment is shown in Table I, and contains virtually all non-electrolytes known to be actively transported.

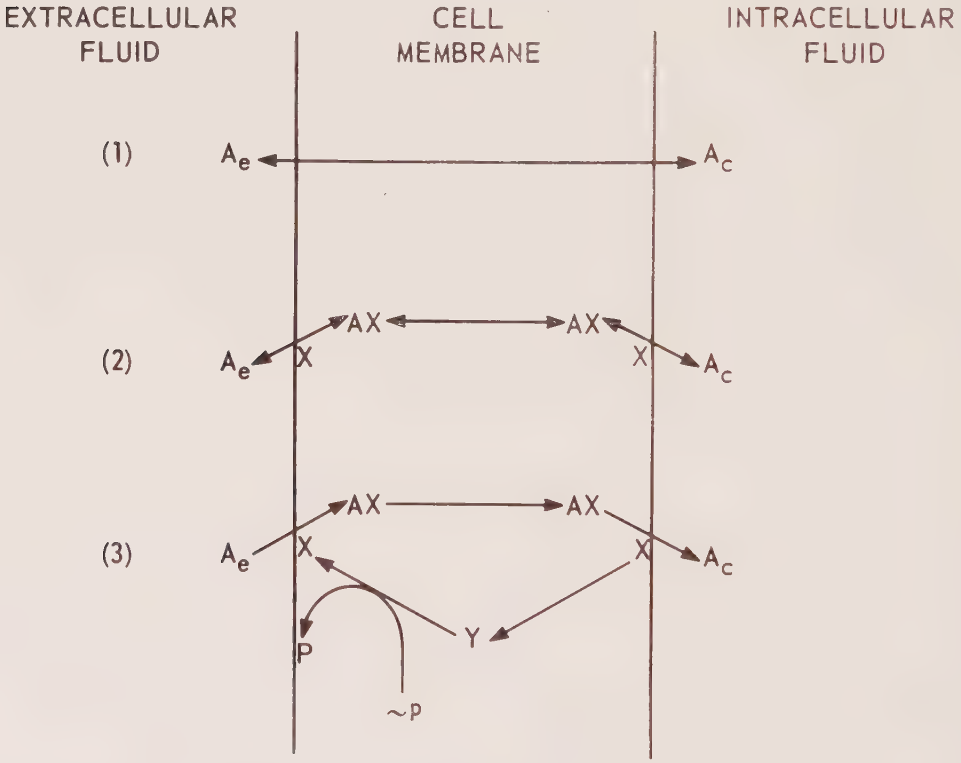
Before attempting to elucidate the role of sodium in the transport process, it may be appropriate to review the present concepts of membrane structure and function. We may then examine effects of sodium ion on different components of the transport process.

TABLE I. MAMMALIAN SYSTEMS REQUIRING SODIUM FOR NON-ELECTROLYTE TRANSPORT

TISSUE	SUBSTANCE TRANSPORTED	REFERENCE
Small Intestine	Glucose	Riklis and Quastel[1]
	Glucose analogues	Bihler and Crane[2]
	Tyrosine	Harrison and Harrison[3]
	Phenylalanine, uracil	Czaky[4]
	*ACPC**, glycine, lysine, methionine	Rosenberg *et al.*[5]
	Taurocholate	Playoust and Isselbacher[6]
Kidney Cortex	Galactose	Kleinzeller and Kotyk[7]
	AIB#, glycine, lysine histidine	Fox *et al.*[8]
Diaphragm Muscle	*AIB*, 2-deoxyglucose	Parrish and Kipnis[9]
Leukemic Leukocytes	*AIB*, glycine, glutamic acid	Yunis *et al.*[10]
Normal Human Leukocytes	Alanine, *AIB*	Rosenberg and Downing[11]
Ehrlich Ascites Tumor Cells	Glycine	Christensen *et al.*[12]
Thymus Nuclei	Amino acids, thymidine, adenosine, adenine	Allfrey *et al.*[13]

*1-amino cyclopentane-5-carboxylic acid
#alpha aminoisobutyric acid

The transport of solutes across the cell membrane is a process fundamental to all biology. Selective transport is the process by which the living cell maintains an intracellular environment, often strikingly different from that in the surrounding extracellular fluid. This process exerts a profound influence on systems responsible for specialization, metabolism, and replication of the cell. A basic factor in this transport is the presence of a plasma membrane at the cell surface which acts as a diffusion barrier. This membrane, according to widely accepted concepts, is composed of protein and micelles of lipid, oriented to contain pores which will permit the passage of only water and small molecules[14, 15]. While lipid-soluble substances cross the membrane by transiently going into solution in the membrane lipid, larger hydrophilic molecules, such as sugars and amino acids, are neither soluble in lipid, nor can they pass freely through the pores. Nevertheless, such non-electrolytes do penetrate cell membranes at rates faster than predicted by diffusion kinetics, and they are concentrated by



(1) = Passive Diffusion
(2) = Carrier Mediated Exchange Diffusion
(3) = Carrier Mediated Active Transport

Fig. 1. Schematic representation of transmembrane transport. "A_e" and "A_c" refer to substrate in extracellular and intracellular spaces respectively. "X" denotes the (mobile) membrane carrier, "AX" the carrier-substrate complex and "Y" the inactivated carrier. Coupling with energy is shown by "~ P→P" reaction.

the cell to produce striking electrochemical gradients at the expense of metabolic energy.

The cellular accumulation of a solute is the resultant or balance of influx and efflux processes. The entry process can, as seen in Figure 1, be divided into three components: passive diffusion, facilitated or carrier-mediated diffusion, and energy-requiring transfer against an electrochemical gradient. These processes occur simultaneously, the prevalence of one or another depending upon the concentration of the transported material in relation to the intracellular content and the availability of carrier sites on the membrane.

Although all living cells are able to accumulate solutes against electrochemical gradients (indeed, this ability may be used to distinguish living from dead cells), in higher forms, the systems which are specialized to perform this transport function for the organism are the epithelium of the small intestine and the proximal tubule of the kidney. We will explore the mechanism of the participation of sodium in active transport by focusing upon studies in these tissues.

In *in vitro* studies, the concentrating ability of any tissue preparation for a given non-electrolyte may be represented by the ratio of the concentration in the intracellular fluid to that in the incubation medium or extracellular fluid. With this distribution ratio, i.e., $\frac{\text{Intracellular fluid concentration}}{\text{Extracellular fluid concentration}}$ the effects of varying sodium concentrations in the incubation medium may be quantitated. In the experiments represented in Figure 2, carbon^{14}-labeled amino acids and an analogue of glucose were used to study sodium ion effects in kidney cor-

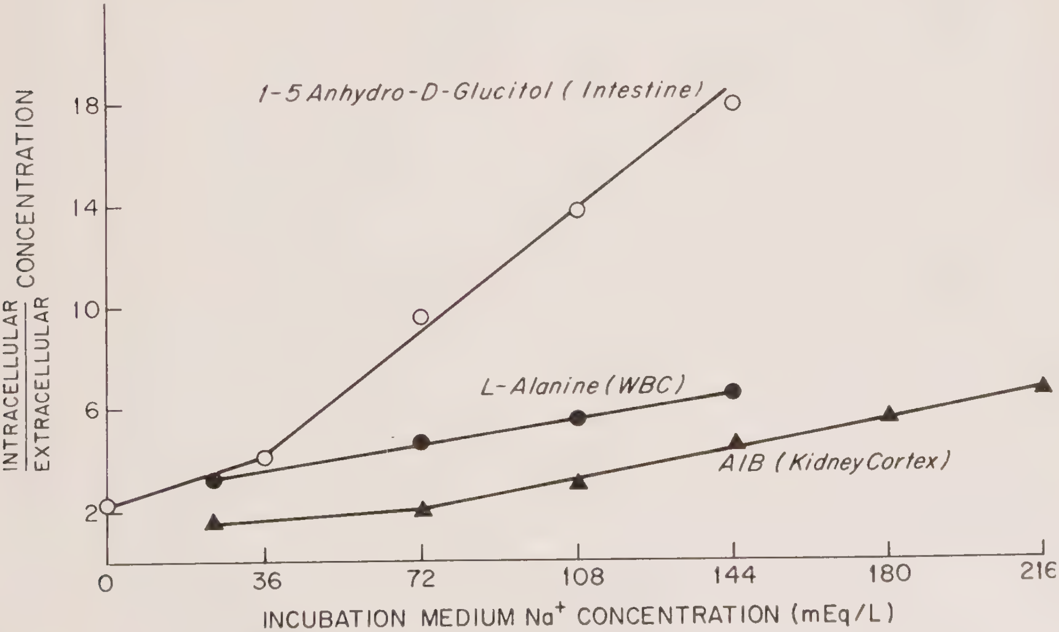


Fig. 2. Influence of medium Na+ concentration on accumulation of sugars and amino acids by mammalian tissues. For details of experimental technique please refer to original publications: anhydro-D-glucitol (2); L-alanine (11); AIB (8).

tex slices[8], normal human leukocytes[11] and in segments of jejunum[2]. The sodium ion effect in kidney was linear even beyond physiologic concentratons of 144 mEq per L. Substitution of lithium, potassium, *TRIS,* lysine, choline, ammonium, rubidium and cesium for sodium ion in these or other systems resulted in a dimunition if not an abolition of the concentration gradient.

EFFECT OF SODIUM ON DIRECTIONAL FLUXES

Sodium could enhance the accumulation of a non-electrolyte either by accelerating the rate of flow into the cell or by slowing the rate of exit from the cell. Czaky found that the influx of sugar into rat intestine was greatly stimulated by sodium ion[4]. Kinetic analysis of the steady state uptake of a non-utilizable amino acid, *ACPC,** by gut segments has been carried out in our laboratory. These studies (Figure 3) demonstrated a striking increase in the influx rate constant in the presence of optimal concentrations of sodium, while effects on efflux were minor. In studies of *AIB*** transport in rat diaphragm, Parrish and Kipnis showed that influx alone was enhanced by sodium[9]. It is, therefore, likely that the sodium ion influences largely the rate of entry of non-electrolytes into the cell, without significant effect on efflux.

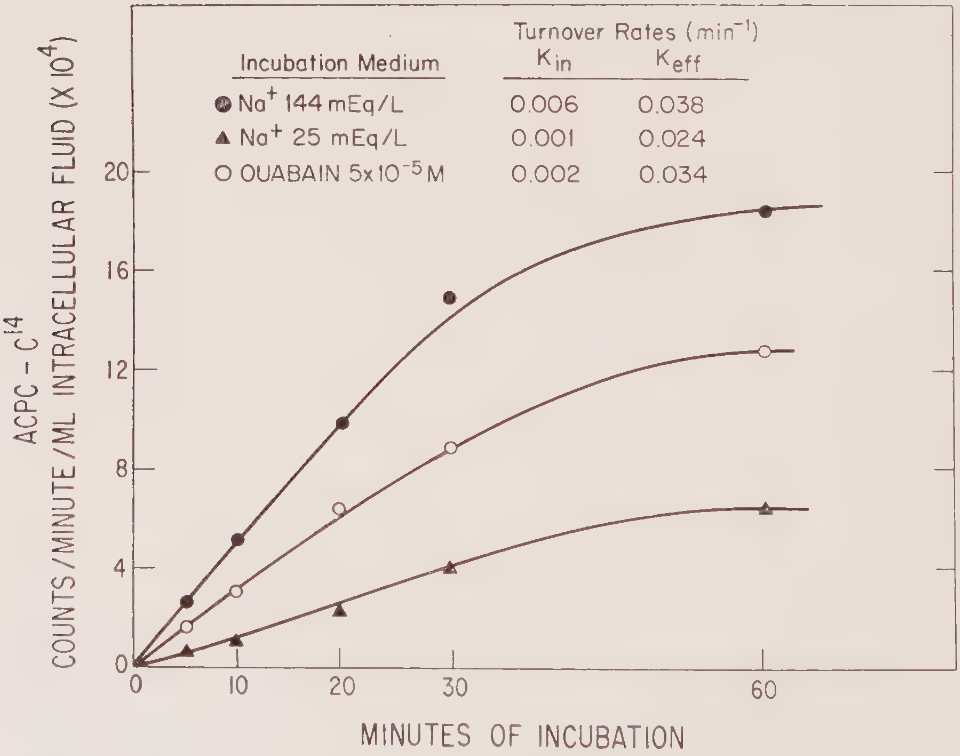


Fig. 3. Kinetic analysis of steady state uptake of ACPC by rabbit intestinal segments. K_{in} and K_{eff} denote rate constants for influx and efflux respectively (data are taken from Rosenberg et al. [5]).

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*1-amino cyclopentane — 5-carboxylic acid.
**alpha aminoisobutyric acid.

Enhancement of the rate of entry could be achieved by any or all of three mechanisms: (a) alteration of membrane configuration to diminish the barrier to diffusion; (b) acceleration of interaction between the transported material and the membrane carrier externally or the release from the carrier internally; or (c) stimulation of cellular processes which provide energy for the accumulation of the transported solute against an electrochemical gradient. It should be emphasized that only the latter mechanism, alone or in combination with the other mechanisms, would result in concentrative transfer, *i.e.*, an intracellular to extracellular concentration ratio greater than one.

SODIUM ION EFFECTS ON THE CARRIER MECHANISM

There is considerable controversy regarding the influence of sodium on non-energy requiring carrier-mediated diffusion. Czaky sought to minimize energy-requiring transport of glucose in guinea pig intestine by utilizing large enough concentrations of sugars to produce a large downhill concentration gradient[16]. Under such conditions the replacement of sodium by lithium or potassium in the perfusing fluid had no effect on the diffusion of glucose. Parrish and Kipnis, using comparably large *AIB* concentrations, made similar observations in rat diaphragm[9]. These workers have interpreted their results as evidence that sodium does not influence carrier-mediated diffusion. We believe that such experiments demonstrate that sodium does not enhance simple diffusion driven by large concentration gradients. Other experimental approaches must be examined for information on carrier-mediated diffusion. Bihler *et al.*[17] found that sodium enhanced the uptake of sugar by intestine under anaerobic conditions when minimal energy would be available to support active transport. They employed glucose concentrations in the range in which carrier-mediated diffusion is operative and concluded that sodium was required for this modality of glucose transport. They speculated that there might be a linked flux of sodium and sugar into the intestinal cell; an hypothesis supported by subsequent work of Schultz and Zalusky[18]. Playoust and Isselbacher found that penetration of rat intestine by bile salts was negligible in the absence of sodium while in its presence serosal to mucosal gradients in excess of one were achieved[6]. In our laboratory we sought to minimize cell metabolism by employing a combination of two metabolic inhibitors, cyanide and fluoride. In agreement with the work of Bihler and Crane, we found that the sodium ion effect was apparent even when active transport was abolished by these inhibitors (Table II). Another experimental design was employed to demonstrate the effect of sodium on the carrier mechanism *per se*. The synthetic amino acid alpha-aminoisobutyric acid is not concentrated by the intestinal cell *in vitro* in Krebs-Ringer bicarbonate buffer[19]. This compound, which penetrates the cell by a carrier-mediated process,[19] reaches higher concentrations in the intestine in the presence of sodium than in its absence (Table II). The weight of evidence, then, points to a role for sodium in carrier-mediated diffusion processes dissociated from energy-dependent transport. Proof of this involvement must await the demonstration of the specific mechanism by which the sodium ion could modify the struc-

TABLE II. INFLUENCE OF EXTRACELLULAR NA^+ ON TRANSPORT OF AIB AND ACPC BY JEJUNAL SEGMENTS

AMINO ACID (mM)	CONDITIONS	INCUBATION MEDIUM Na^+ (mEq./L.)	$\frac{ICF}{ECF}$
AIB (0.2)	Aerobic	25	0.06 ± 0.015
		144	0.22 ± 0.046
ACPC (0.12)	CN^-, F^- inhibited*	25	0.47 ± 0.09
		144	0.91 ± 0.06

Segments were incubted for thirty minutes at 37° C. following a fifteen minute preincubation.
*CN^- concentration — 5×10^{-3} M.
F^- concentration — 10^{-2} M.

ture or configuration of the membrane to facilitate the carriage or translocation of the non-electrolyte into the cell.

SODIUM EFFECTS ON ACTIVE ENERGY-LINKED TRANSPORT

There is more agreement among investigators concerning the role of sodium in the energy-requiring active transport process. This active process might be termed a non-electrolyte pump analogous to the energy-requiring pump involved in the transport of sodium. Since all non-electrolyte pumps are inhibited by the removal of sodium from the system, it is clear that the sodium effect is non-specific and functions in combination with many separate membrane carrier systems.

IMPORTANCE OF INTRACELLULAR CATION CONCENTRATION

Riggs *et al.*[20] suggested that intracellular potassium levels are crucial to active transport. In studies of intestinal transport we measured intracellular sodium and potassium concentrations after incubation in buffers of varying sodium concentration. It can be seen in Table III that the intracellular content of both these cations rises with increasing extracellular sodium. The increasing potassium levels are, no doubt, the reflection

TABLE III. INFLUENCE OF MEDIUM SODIUM CONCENTRATION ON TISSUE CATION CONCENTRATION

INCUBATION MEDIUM Na^+ (mEq./L.)	TISSUE CATION CONCENTRATION mEq./kg. WET WEIGHT		
	Na^+	K^+	$Na^+ + K^+$
25	25.4 ± 2.3	39.2 ± 4.3	64.6
72	48.7 ± 1.3	44.8 ± 2.4	93.5
108	65.3 ± 5.6	50.3 ± 5.2	115.6
144	89.7 ± 1.7	59.1 ± 4.4	148.8

Data were obtained from experiments with rabbit jejunal sacs incubated for ninety minutes. Cation concentrations are expressed as mean ±S.D. and represent a minimum of four experiments.

of improved cation pump at the higher extracellular sodium concentrations. The possibility that the ratio of these cations across the membrane or within the cell is the controlling factor in active transport has yet to be tested.

RELATIONSHIP BETWEEN TRANSPORT OF SODIUM AND NON-ELECTROLYTES

We have reviewed the evidence which indicates the importance of the sodium ion in non-electrolyte transport. That this stimulatory effect of the sodium ion might be intimately linked to active cation transport was first suggested by Czaky[4]. He reported that ouabain, a known inhibitor of the cation pump, also inhibited intestinal transport of sugars, amino acids and pyrimidines. This effect of ouabain has been confirmed in many laboratories, including our own (Figure 4) and in many tissues. Further evidence for a link between sodium transport and non-electrolyte transport came from work of Schultz and Zalusky[18] who demonstrated that active sodium transport was stimulated by active transport of sugar or amino acid in rat ileum, and from studies in our laboratory (Figure 5) which showed

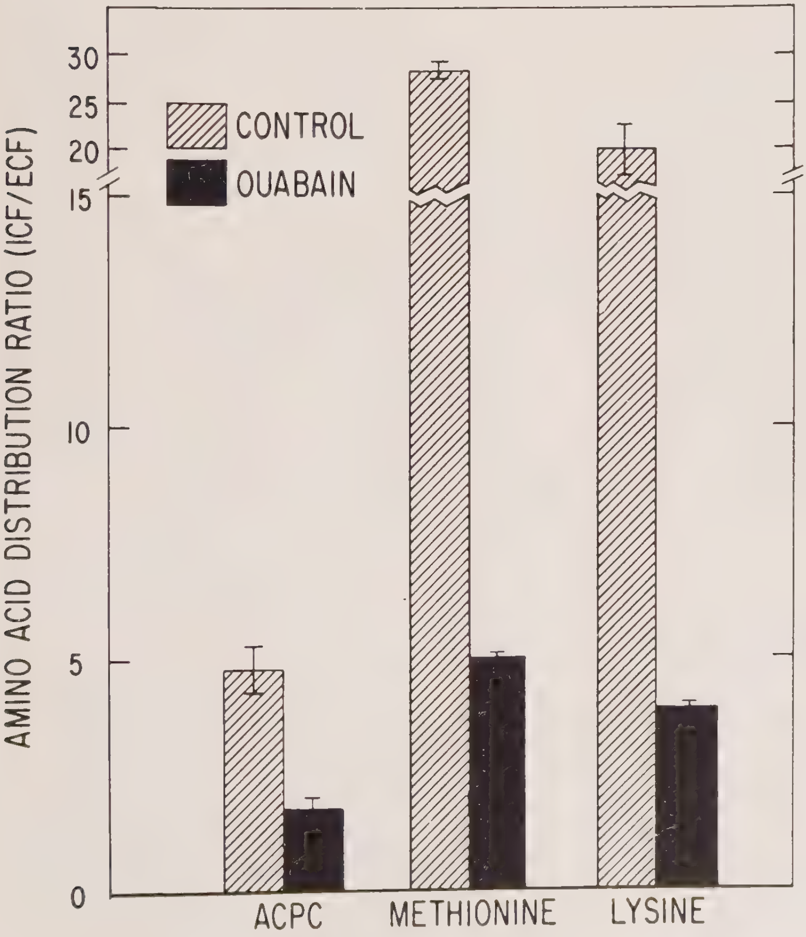

Fig. 4. Inhibition of amino acid accumulation in rabbit intestine by ouabain (5×10^{-5}M). Everted jejunal segments were incubated at 37° in Krebs-Ringer bicarbonate buffer, pH 7.4.

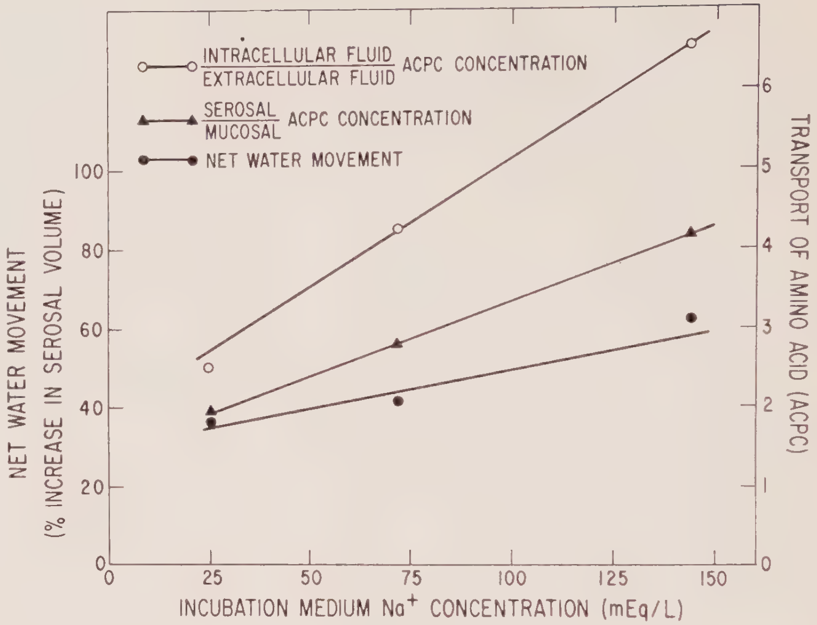

Fig. 5. Enhancement of active transport of sodium and ACPC by increasing incubation medium Na+ concentration. Sodium transport was estimated by measuring net water movement into everted jejunal sacs (from Rosenberg et al. [5].)

that increasing medium sodium concentration resulted in parallel enhancement of active sodium transport and amino acid transport in everted intestinal sacs. Although there is no direct evidence which bears on the biochemical mechanism of this relationship, current interest is focused on membrane adenosine triphosphatase.

MEMBRANE ADENOSINE TRIPHOSPHATASE *(ATPase)*

In 1957 Skou[21] described a sodium and potassium stimulated, magnesium dependent ATPase in crab nerve membrane. On the basis of the striking cationic requirements of this enzyme (or group of enzymes), he predicted that this *ATPase* would be shown to be associated with the conversion of chemical energy from *ATP* into osmotic energy for active cation transport. A similar, if not identical enzyme system has subsequently been identified in the cell membrane of virtually all tissues capable of active transport. Several lines of evidence have supported the thesis that this Na-K stimulated *ATPase* is intimately related to, and may indeed control active transport processes. Blond and Whittam[22] have shown recently a direct relationship between *ATPase* activity and cell respiration. These workers suggested that the *ADP* produced in the course of *ATP* hydrolysis might be the "pacemaker" of cell respiration, and thus provide the essential link between energy metabolism and transport. It remains to be seen whether this enzyme system will also be shown to provide the link between the transport of cations and organic non-electrolytes such as sugars and amino acids.

POTENTIAL CLINICAL SIGNIFICANCE

We have presented evidence that sodium is involved in non-energy dependent carrier-mediated diffusion as well as energy-requiring active transport.

Although the clinical significance of this finding has yet to be demonstrated, numerous possibilities present themselves. In hyponatremic states associated with cardiac failure, cirrhosis, and salt depletion, there exists the potential for significant cell dysfunction related to impaired transport mechanisms. We need not stretch speculation too far to suggest that the wasting and inanition found together with chronic hyponatremic may be in part the result of diminished transport of non-electrolytes crucial to cell metabolism and protein synthesis.

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Regulation of Calcium and Phosphorus Metabolism*

LEO LUTWAK, Ph.D., M.D.

The regulation of calcium and phosphorus metabolism may be equated with the control of skeletal physiology, since approximately 99 per cent of the body calcium is present in the bone associated with an equivalent amount of phosphorus. The remaining 1 per cent of the body calcium is found in the circulating extracellular fluid where calcium plays a significant role in tissue permeability, membrane transport, blood coaguability, neuromuscular irritability, and probably other fundamental physiologic phenomena. In man, the concentration of free ionic calcium in the extracellular fluid is maintained within narrow limits at a level of approximately 2.5 mEq. per L. There is an approximately equal amount present in the circulation in the form of non-ionized, primarily protein-bound calcium. The homeostatic concentration of this cation is maintained by the action of the various endocrine systems, the kidneys, and the gastrointestinal tract (Fig. 1).

The endocrine systems implicated in the regulation of the critical level include the pituitary, the thyroid, parathyroids, adrenals and gonads. Calcium is being removed from the extracellular fluid under normal conditions at a relatively constant rate through mechanisms of renal excretion and endogenous fecal excretion. In addition, during lactation, a significant amount of calcium is also lost from the body. During pregnancy, calcium leaves the body to be incorporated into the fetus. Body calcium can be replenished only by absorption from the gastrointestinal system. When mechanisms of absorption are abnormal, or when the amounts of dietary calcium available for absorption are insufficient, calcium concentration in the circulating fluids of the body must be maintained by resorption from the skeleton. Thus, disorders of calcium and phosphorus metabolism can result in demineralization of the skelton and may be attributable to the action of the endocrines, to defects in dietary intake, to abnormalities of mechanisms of absorption, or to abnormalities in excretory patterns[14].

Phosphorus, in addition to being present in the skeleton as part of the calcium hydroxyapatite crystals of bone mineral, is also present in all of the cells, intimately related to energy transformation reactions. Phosphorus metabolism may be affected specifically by the various endocrine systems as well as following effects on calcium metabolism indirectly.

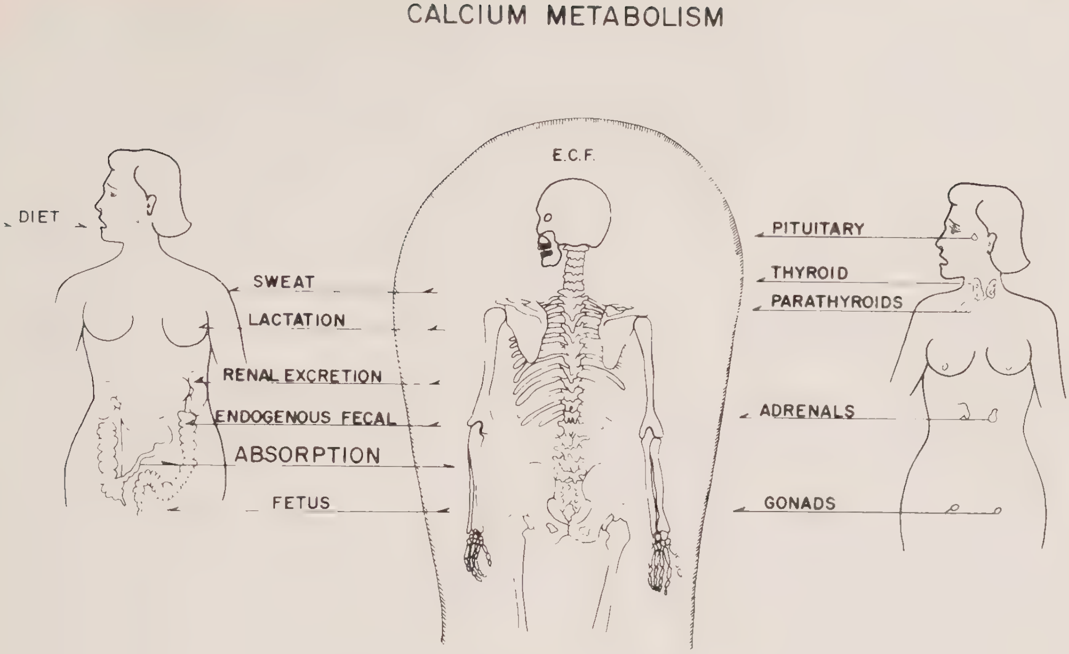

Fig. 1. Calcium Homeostasis reprinted from Lutwak[15].

The regulation of calcium and phosphorus metabolism, therefore, is a function of the activity of endocrine systems, the renal tract, and gastrointestinal tract.

ENDOCRINE CONTROL

Pituitary. Studies in patients with acromegaly have demonstrated two types of effects on calcium metabolism resulting from excessive growth hormone. Growth hormone produces increased growth of all components of the body including the skeleton. This results in increased demands for calcium and phosphorus as evidenced by the presence of hyperphosphatemia. In addition, human growth hormone has been demonstrated to have a hypercalciuric effect independent of renal function. The hypercalciuric phenomenon requires the presence of the parathyroids or of sufficient vitamin D, suggesting that this is a direct action on the skeleton mediated through parathyroid function[17, 20]. In the absence of the anterior pituitary, i.e., in hypopituitarism, no defects of calcium or phosphorus metabolism have been described.

Thyroid. In hyperthyroidism, generalized hypermetabolism involves all systems including the skeletal, renal, and gastrointestinal. As a result, increased absorption of calcium and phosphorus is usually seen, as well as increased renal excretion of calcium and phosphorus, and increased bone breakdown. Occasionally, hypercalcemia has been reported in association with florid hyperthyroidism. Marked bone demineralization can occur in long-standing hyperthyroidism, particularly when dietary calcium has been inadequate. When hypercalcemia is associated with hyperphosphaturia, which may result from rapid tissue breakdown, the differential diagnosis between hyperthyroidism and hyperparathyroidism becomes of importance. Conversely, in myxedema, there is decreased turnover of the the skeletal system as demonstrated by the use of radioactive isotopes, as well as decreased absorption of calcium and phosphorus from the intestine. No significant bone pathology has been reported in chronic hypothyroidism, however, nor any significant defects in serum calcium or phosphorus concentrations.

Adrenal Cortex. Hyperadrenocorticism, whether due to intrinsic disease or secondary to therapy with exogenous corticosteroids, has long been implicated in calcium and phosphorus metabolism. It was suggested by Albright that a relative excess of the antianabolic activities of the adrenal cortical hormones would lead to skeletal demineralization, and as a matter of fact, this was considered the primary cause of osteoporosis[19]. Studies with radioactive tracers have demonstrated that adrenocortical steroids inhibit both the rate of bone formation and of bone resorption[13]. In many individuals, bone formation is inhibited to a greater extent than is resorption with the result of decrease in bone mineral. Adrenocortical steroids have a primary action in reducing tubular reabsorption of many substances including calcium and phosphorus, thus resulting in excess loss of these elements in the urine. On the other hand, the action of the adrenocortical steroids on the gastrointestinal absorption of calcium is less constant. It has been demonstrated that in some individuals, these hormones will produce a decreased absorption of calcium; on the other hand, in others, adrenocortical steroids will promote better absorption of calcium[23].

Gonads. Observations of Pfeiffer and Gardner[18], in the 1930's demonstrated that in avian species, estrogens promoted the deposition of mineral into bone. Relative estrogen lack produced by castration resulted in decreased mineral of the skeleton in chickens and pigeons. In other species, the action of the estrogen have been much less clearly defined. Neither estrogen nor androgen have been reported to produce a rise in serum calcium in mammals. In man, both estrogen and androgen result in decreased urinary excretion of calcium; and in some individuals these agents have been demonstrated to produce increased absorption of both calcium and phosphorus from the gastrointestinal system. Androgenic substances, because of their specific action on protein synthesis, have been shown to increase the retention of phosphate in amounts comparable to the nitrogen being stored.

Parathyroid. The parathyroid hormone is the one endocrine substance with a specific action on calcium and phosphorus metabolism. Purified preparations of parathyroid hormone demonstrate direct action absorption of calcium from the intestine[21], reabsorption of phosphate by the renal tubules[5, 10], and both in bone formation and bone resorption[1, 3]. In hyperparathyroidism, resorption from the skeleton is increased, absorption of both calcium and phosphorus from the gastrointestinal tract are increased, and tubular reabsorption of phosphate is markedly decreased. (Whether or not the parathyroid hormone has a specific action on renal handling of calcium is still open to question.) As a result, in hyperparathyroidism an elevation of serum calcium and a depression of serum phosphate is usually seen. Both hypercalciuria and hyperphosphaturia may be present. Conversely in hypoparathyroidism, because of the lack of fine control of calcium concentrations, serum calcium is markedly decreased, serum phosphate is elevated, and both urinary calcium and phosphorus decreased.

The serum calcium concentration in hypoparathyroidism is very close to that seen in *in vitro* systems of bone and serum, suggesting that a concentration of 3 to 3.5 mEq. per L. of calcium represents solubility of bone in the absence of physiological control and that the parathyroid is thereafter responsible for fine adjustments to the homeostatic level of 5 mEq. per L. Recently, the action of a second hormone, calcitonin, responsible for decreasing serum concentrations of calcium has been demonstrated[2, 4, 9]. Thus, a "push-pull" system for the fine control of calcium concentration in extracellular fluids is available, with parathyroid hormone causing elevation and calcitonin depression of calcium levels.

RENAL TRACT

In the kidney, calcium is filtered at the glomerulus and reabsorbed by the tubules. Thus, intrinsic renal pathology leading to disturbances at either of these anatomical sites will affect the homeostasis of both calcium and phosphorus metabolism. Renal tubular disease, congenital and acquired, leads to the so-called hypophosphatemic vitamin D refractory rickets. The primary defect appears to be a renal tubular lesion leading to excessive phosphate clearance, with the development of secondary hyperparathyroidism and the clinical picture of rickets, osteitis fibrosa, and osteomalacia. A similar clinical syndrome may result in chronic azotemic renal failure[12].

GASTROINTESTINAL SYSTEM

Chronic absorptive disorders of the gastrointestinal system, such as sprue, result in malabsorption of calcium, phosphorus, and vitamin D. The usual clinical syndrome is that of osteomalacia, frequently with decreased serum calcium and phosphorus and hypocalciuria and hypophosphaturia. In sarcoidosis, on the other hand, the gastrointestinal system demonstrates increased sensitivity to vitamin D with resulting increased absorption of calcium, the development of hypercalcemia and hypercalciuria.

PRIMARY NUTRITIONAL DISORDERS

Phosphate deficiency does not occur in man because of the widespread occurrence of this element. Calcium deficiency, on the other hand, has been described frequently and in recent years as being considered a primary cause of osteoporosis in some individuals[11, 16,22]. Dietary deficiency of calcium, as a rule, does not result in measurable changes in serum calcium. After the ingestion of a large bolus of calcium, slight evanescent rises in serum calcium have been demonstrated[6]. The normal homeostatic control, however, usually prevents these from being of significant magnitude. In various species such as the monkey, the cat, and the horse, high dietary phosphate regimens have been shown to produce a form of secondary hyperparathyroidism, which after long periods of time, may result in hypercalcemia and osteitis fibrosa[7, 8].

The role of vitamin D in the control of calcium and phosphorus metabolism has been related to the mechanism of action of the parathyroid. Vitamin D produces elevation of serum calcium, hyperphosphaturia (through decreased tubular reabsorption of phosphate), both increased bone formation and increased bone resorption, as well as increased intestinal absorption of both calcium and phosphorus.

Other nutrients that have been implicated in the control of calcium and phosphorus metabolism include vitamin C, dietary protein, lactose, fluoride, copper, magnesium, and fat.

SUMMARY

The regulation of calcium and phosphorus metabolism has been reviewed briefly and the roles of the endocrine system, gastrointestinal system, renal system, and diet have been outlined.

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Regulation of Magnesium Metabolism

DONALD P. TSCHUDY, M.D.

The physiologic functions of magnesium appear to be related to the activation of a number of enzyme systems and to the control of neuromuscular excitability. It has been shown that magnesium is capable of activating a number of enzyme systems *in vitro,* including phosphatases, transphosphorylases, pyrophosphatases, carboxylases, hexokinase, enolase, and others[1]. The action of magnesium is not always specific in that other metals may produce similar activation. Some activity is usually present in the absence of magnesium. The neuromuscular effects of magnesium and calcium involve three areas: nerve conduction, transmission at the myoneural junction, and muscle contraction.

The effects on nerve conduction of changes in both magnesium and calcium concentrations are similar. A decreased concentration of calcium and/or magnesium produces increased irritability of motor nerves as a result of a lowering of the threshold to stimulation. An increase of either ion increases the excitatory threshold[2, 3,4]. The effects at the myoneural junction are complex, since the overall effect is the result of the sum of the membrane charge effect and the effect on the liberation of acetylcholine[5–8]. The net effect of a decrease in the concentrations of magnesium or calcium is an increase in transmission at the motor end plate. In the case of magnesium, decreasing concentrations lower the excitatory thresholds of nerve and muscle membrane and also increase the liberation of acetylcholine. Thus, the summation of all three effects increases transmission of the nerve impulse. While lowered concentrations of calcium have the same effect on both nerve and muscle membrane as lowered concentrations of magnesium, the effect on the release of acetylcholine is the opposite. Thus, decreased calcium concentrations increase nerve and muscle excitability but decrease the release of acetylcholine.

The total body content of magnesium is about 20 to 30 grams[9, 10], of which about one-half is present in bone. About 0.5 grams is present in extracellular fluid, and the rest is present intracellularly in soft tissues. Intracellular magnesium is second in concentration to potassium, the intracellular level being about ten times the plasma concentration[11]. Studies of the kinetics of magnesium distribution have been limited to a twenty-four-hour period because of the short half-lives of the radioactive isotopes of magnesium. The exchangeable pool at twenty-four hours is only slightly greater than the amount of magnesium in the extracellular fluid[12–14], suggesting that most of the body magnesium is not in rapid equilibrum with the extracellular magnesium.

The normal concentration of magnesium in serum by the multichannel flame spectrometer method reported by Vallee *et al.* is 2.0 + .16 (SD) mEq. per L.[15]. Magnesium exists in the plasma as both an ionized form and a fraction which is protein bound, the latter comprising about 35 per cent of the total[16–18]. Studies with purified proteins suggest that the dissociation constant of magnesium protein complexes is the same as that of the corresponding calcium protein complex[19, 20]. This would suggest that the ratio between bound and total serum magnesium would remain relatively constant over a range of values of total serum magnesium, as has previously been demonstrated for calcium[21]. Alterations in plasma proteins, however, do cause changes in the proportion of bound magnesium. Nomograms have been prepared by Willis and Sunderman by which corrections can be made for the total protein[22]. Small amounts of non-ionized magnesium are maintained in solution in the plasma as soluble complexes with compounds such as citrate, the physiologic significance of which is unknown.

The daily requirement of magnesium is not certain, but balance has been maintained in normal individuals with an intake of 220 mg. (18 mEq.) daily[23]. A normal diet contains about 20 to 40 mEq., of which about one-third or less is absorbed. Fecal magnesium is primarily that which has not been absorbed from the diet[14, 23, 24, 25]. Urinary excretion of magnesium in twelve normal men averaged 162 ± 45 mg. (13 mEq.) per twenty-four hours[26]. Since only 4 to 6 per cent of the magnesium filtered at the glomerulus is excreted, it is clear that magnesium undergoes extensive tubular reabsorbtion[28]. In normal individuals, renal conservation of magnesium appears to be efficient, since administration of a magnesium deficient diet for a 3 to 4-week period in two normal men resulted in no decrease of serum magnesium and total losses of 25 and 72 mEq.[29]. Urinary excretion of magnesium decreased markedly after the curtailment of magnesium intake.

The first demonstration of the effects of magnesium deficiency was the observation in 1932 by Kruse *et al.* that deprivation of this cation produced tetany and convulsions in rats[30]. Similar phenomena were later shown in other species following magnesium deprivation[31–33]. Tetany resulting from magnesium deficiency occurs spontaneously in cattle, a condition known as "grass staggers"[34–37]. Convincing evidence has recently been presented that tetany in humans resulting from magnesium deficiency is a specific clinical entity resembling hypocalcemic tetany[15]. The two can be differentiated only by chemical means. A number of neuromuscular abnormalities have previously been observed in patients with hypomagnesemia[38–42], including muscular twitching and tremor, sweating, tachycardia, anxiety, delerium with hallucinations, confusion, disorientation, convulsions and coma. The exact relationship of some of these symptoms to hypomagnesemia is uncertain in view of the fact that hypomagnesemia has been observed in the absence of clinical manifestations[43]. However, in the patients observed by Vallee *et al.* conclusive proof that the syndrome of tetany (including spasms of facial and extremity muscles, convulsions and laryngeal stridor) can result from magnesium depletion is "afforded by the correlation of symptomatology with the chemical changes in serum, and of the concomitant restoration of both to the normal state by therapy"[15].

Since simple restriction of magnesium intake does not produce tetany[29, 44], it would appear that multiple factors are involved in the etiology of hypomagnesemic tetany. These include excessive loss from the gastrointestinal or urinary tract coupled with diminished intake of magnesium. All patients have had a debilitating disease such as prolonged acute infection, alcoholism with malnutrition and gastritis, intestinal obstruction with postoperative drainage of intestinal contents[15], and acute porphyria[45]. The prolonged administration of parenteral fluids without magnesium supplementation probably plays a significant etiologic role. Other conditions in which a decreased concentration of serum magnesium has been reported include epilepsy, delerium tremens, cirrhosis, congestive heart failure, nephritis, eclampsia, pancreatitis, hyperparathyroidism and hyperaldosteronism[26, 27].

The effects of experimental hypermagnesemia include transient tachycardia, followed by bradycardia and depression of atrioventricular and intraventricular conduction as the concentration of magnesium rises[46]. Peripheral vasodilatation with a fall in blood pressure is observed. Depression of the nervous system is also a result of excess magnesium ion. The first manifestation of serious magnesium toxicity is probably areflexia[1]. Drowsiness and coma may occur with excess magnesium as evidenced by the appearance of these signs in patients with chronic renal insufficiency who were given magnesium sulfate orally[26, 27]. Hypermagnesemia is seen most frequently in patients with acute renal failure and sometimes in chronic renal disease with uremia and oliguria[43, 47–49]. It also may occur in diabetic coma, prior to therapy[43, 50], oxalic acid poisoning and severe dehydration[1].

An understanding of the physiology and pharmacology of magnesium facilitates the early detection and therapy of abnormalities of this ion.

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Regulation of Chloride Metabolism

G. W. SMETTERS, M.D., J. C. SHERRICK, M.D., and
J. de la HUERGA, M.D., Ph.D.

INTRODUCTION

The mechanisms for anion control seem unexciting when contrasted with the endocrine regulation and oxidative transport mechanisms associated with metabolism of cations[10, 19]. Of the anions, chloride at first seems particularly prosaic, since it appears to lack the role played in energy metabolism by phosphate, in hormone formation by iodide, and in protein metabolism by sulfate. It even lacks the glamor of being a trace element. Nonetheless, the chloride ion is an indispensible component of all tissues.

Although most areas of cation metabolism have been extensively investigated, many gaps in knowledge about chloride exchanges still exist. This is surprising, since the attention of earlier biochemical investigations was directed largely toward chloride and other anions rather than cations. Such a situation developed because fairly accurate methods for chloride analysis were available for many years, while the technics of cation analysis were cumbersome and laborious prior to the advent of colorimetric methods and flame photometry.

CHLORIDE BALANCE

Intake: In the normal mammal, all chloride is taken in orally as a component of the diet. Estimates indicate that the usual American diet provides an average of 180 mEq. of chloride ion per day with a range of 70 to 415 mEq.[3, 4]. For balance to exist, losses must be restricted to this amount; if more is lost, it must be replaced by supplementary oral or parenteral intake to prevent rapid development of deficiency.

Loss: Chloride is lost from the body via three main routes: (a) skin, in sweat, tears, etc.; (b) feces, from gastrointestinal secretions and failure to absorb all the chloride ingested; (c) urine. In addition, there is chloride in milk, and this ion is lost along with other anions during menstruation and bleeding.

While sweat is being formed within skin glands, some conservation of ionic content occurs, but once the secretion reaches the skin surface, its contained minerals are lost. In both intestinal contents and urine, however, chloride ion which has been passed to the *outside* of the body is exposed to *reclamation* mechanisms which permit recovery of most of the ion which has been expelled, thus conserving stores[16]. Sweating is rarely completely absent, and the renal and intestinal conservation mechanisms fall short of complete recovery. As a result, a minimum, irreducible loss of chloride, estimated at 1.7 to 8.0 mEq.

per day[4], occurs, even in a fasting individual under basal conditions. This must be supplied by mobile extracellular chloride.

CHLORIDE TRANSPORT

From the site of intestinal absorption, chloride is distributed ubiquitously to all body tissues through the plasma and extracellular fluid[3]. In general, it diffuses readily in the extracellular fluid, although in connective tissue some chloride is bound to collagen[12]. In its distribution, chloride is closely associated with sodium. Both sodium and chloride are largely extracellular ions. In the past, it was thought that chloride might be completely extracellular, leading to the concept of a *chloride space* as a measure of extracellular fluid volume[3]. Both sodium and chloride enter cells, but the amounts retained within the cell are very small compared to the extracellular concentration[3]. Sodium and chloride diffuse into the cell, but active, oxidative, cellular metabolism releases energy which is then used to carry sodium back out of the cell in the process of *active transport* or *redox pumping*[2, 9]. This extrusion of sodium produces an electrochemical gradient from the inside of the cell to the outside. Intracellular chloride then travels passively along this gradient to the outside. In addition to following sodium, chloride ion may move along the gradient established by transport of any other cation out of a cell[3].

Some instances of apparently active transport of chloride itself have been reported in mammals, as in the ileum, sublingual salivary gland, skin after adrenalin stimulation[9] and renal tubule[20]. In most cases, however, the chloride follows passively behind some cation which is being actively ejected. Its general role appears to be to go where necessary to maintain electrochemical neutrality when active cation exchanges occur. Thus, most regulation of chloride movements is indirect, being exerted on the transfers of cations which in turn produce shifts in chloride ion.

Other anions, particularly bicarbonate, also participate in this process of electrochemical *neutralization*, and reciprocal relations between chloride and bicarbonate exist in many areas[10]. Since chloride is neither produced nor consumed by oxidative metabolism, it is sometimes called upon to shift location and compensate for deficits or excesses of more labile anions. Since the amount of bicarbonate present at a given point is closely related to pCO_2 and pH, changes in these factors in tissues and lung cause bicarbonate shifts in and out of cells which are accompanied by chloride transfers[5].

An example is the erythrocyte *chloride shift*[21]. In tissues, metabolism produces CO_2 which diffuses into the blood and elevates the pCO_2. A minute amount of the gas remains dissolved in the plasma, forming bicarbonate; some forms carbamino compounds with plasma proteins, but most enters the erythrocytes. Here some of the CO_2 forms carbamino compounds with reduced hemoglobin, but most is hydrated by carbonic anhydrase to form H_2CO_3 which is dissociated as H^+ and HCO_3^-. The H^+ ions are buffered by the hemoglobin; the HCO_3^- which is left diffuses out into the surrounding plasma. Cations apparently cannot leave the erythrocyte readily to balance this discharge of bicarbonate. Instead, to balance the electrochemical gradient so established, chloride ions move into the erythrocyte[5]. In the lungs, the process is reversed. The pCO_2 in the plasma falls as dissolved CO_2 leaves to enter the alveolar lumen. Bicarbonate then combines with H^+ to form H_2CO_3 which is dehydrated to release more CO_2; this also leaves. Since bicarbonate is more plentiful in plasma than within erythrocytes, it might be expected that the plasma bicarbonate would be depleted first. However, the erythrocyte contains much more carbonic anhydrase than plasma and can dehydrate bicarbonate much more rapidly. Consequently, CO_2 is released much faster within the cell than outside it. As this CO_2 leaves, the intracellular bicarbonate level falls and more HCO_3^- enters from the plasma to be dehydrated. This process produces a surplus negative charge within the cell, and chloride shifts back out into the plasma to compensate for it[5].

CHLORIDE EXCHANGES WITH THE EXTERNAL ENVIRONMENT

Although chloride moves between cells and extracellular fluid in all areas, the most striking and significant exchanges occur at points where this ion enters or leaves the body, i.e., skin, mammary gland, gastrointestinal tract, and kidney.

Sweat Chloride: Sweat production is predominantly concerned with water flow, and is controlled by mechanisms reflecting skin and body temperature, and external environmental conditions. The mineral content of sweat is quite variable, and depends on availability of salt stores, the rate of sweat production, and the functional capacity of the sweat glands[16]. The endocrine influence on sweat composition is well known[10, 11, 16]. Conservation mechanisms decrease mineral content in cases of salt deficiency. In all of these mechanisms, control seems to be exerted directly on sodium reabsorption within the sweat gland. Chloride enters the sweat in a passive manner along with the sodium[10]. In the case of strong stimulation, as in epinephrine application, some data indicate a possible active transfer of chloride itself[16] but this is under unusual conditions.

Milk Chloride: During lactation, the volume and composition of milk depends on nervous and hormonal regulation, general nutrition, and particularly on the demand. Together with other ions, chloride is lost in this way. Again, it appears to be a passive component, following behind active cation secretion. During late pregnancy, the chloride balance is positive and is above the demands of the fetus; however, during lactation, chloride balance becomes negative and may remain so after lactation has ceased[16].

Gastric Chloride: Food in the stomach and upper intestine stimulates gastric secretion directly, by neural reflex, and by endocrine mechanisms[7, 8]. In addition to enzyme production, this stimulation is thought to lead to active metabolism in parietal cells with production of CO_2 which is hydrated to form H_2CO_3[10]. The H^+ ion is actively transported into the gastric lumen[3, 10] while the HCO_3^- is carried away in the plasma along with sodium. In addition, sodium and potassium are secreted into the lumen in amounts varying with the secretory rate[14]. Chloride is also released into the lumen to comprise most of the anionic content of gastric juice. Its concentration usually equals that of hydrogen, sodium and potassium combined[14]. The chloride concentration varies much less with flow rate than do the concentrations of the cations[14]. Some of this chloride transfer might be passive, accompanying the cation movements, and some of it might be by exchange diffusion[3]; however, the concentration gradient indicates that at least part of the chloride is carried into the lumen by active transport[3, 7,10]. Whether or not there are separate specific *pumps* for hydrogen and chloride ions or a single combined *pump* for hydrochloric acid is still open to question[8].

Intestinal Chloride: In addition to the chloride which has been ingested and that released into gastric juice, this ion also enters the lumen of the gastrointestinal tract in salivary secretions, bile, pancreatic juice, and the secretions of the upper small intestine[16]. In all of these, the stimulus for release of minerals may be the direct presence of food, neural reflex or endocrine activity[16]. In these areas, chloride enters the secretion chiefly by passive transfer along the electrochemical gradient resulting from direct, active secretion of sodium or other cations[10, 16, 19,22]. In the sublingual gland, however, an electric potential has been found which is abolished by substitution of nitrate for chloride[10], suggesting that some active transport of chloride takes place in this area[9, 19].

Throughout the length of the intestine, chloride and other ions are exchanged between the mucosa and the luminal contents by diffusion[19]. In addition, some evidence of active chloride transport has been reported[8, 19]. The chloride loss in feces is very small and usually not related to oral intake of chloride[4] because of a net absorption of chloride in the lower intestine and colon. Chloride absorption can occur here with elevation of serum levels even though the effect is detrimental to the animal[1]. In uretero-colic anastamoses, chloride may be reabsorbed from the lumen to the point of producing hyperchloremic acidosis[1]. Although this might be due to active chloride transport, it can also be explained by release of large amounts of sodium bicarbonate from the intestinal mucosa into the lumen, followed by sodium reabsorption accompanied by passive uptake of chloride[1].

Renal Chloride: In sweat production, the net effect is loss of chloride. In the gastrointestinal tract, the net effect by the time passage has been completed is uptake of chloride. The real center of chloride control is the kidney, where this ion may be conserved or eliminated so as to maintain concentrations of serum chloride within narrow limits. Since most chloride is extracellular and freely diffusible, it is readily accessible to direct, rapid renal control, unlike intracellular ions such as potassium. Since it is so largely extracellular, direct, rapid control is necessary. No intracellular storage pool is available to replace losses or take up excesses.

In the kidney, chloride again follows the general pattern of passively following cations[6, 15]. Filtration by the glomerulus produces a solution resembling plasma in its chloride concentration. As this solution moves along the proximal tubule, sodium is absorbed by active transport against chemical gradients and at least in part under hormonal control[6, 15, 18, 20]. Both water and chloride follow the sodium back into the renal parenchyma[6, 18]. Since this chloride movement does not always follow the pattern of simple diffusion, a process of exchange diffusion involving a carrier to convey the chloride ion has been postulated[15]; however, this process is still passive, rather than active, chloride transport. A similar series of events is believed to exist in the loop of Henle. In the distal tubule and collecting system, sodium can be strongly reabsorbed and also exchanged for potassium or hydrogen ions[10, 15]. This absorption of sodium produces an electrochemical gradient for passive transfer of chloride out of the lumen. Measurements of this gradient in the distal collecting system indicate that it is still not always strong enough to account for all the chloride reabsorption which takes place, and a process of active chloride transfer in the distal part of the system cannot be excluded[10, 15, 18].

While sodium is being reabsorbed, potassium, hydrogen, ammonium and other ions are being released into the urine. Enough chloride remains behind in the lumen to balance the cations present, although the greatest part of the chloride filtered is absorbed. Renal control of chloride thus consists in regulating the amount of chloride reabsorbed from the filtrate. This regulation is largely indirect. Other ions, chiefly sodium, are directly affected by the renal mechanisms of reabsorption or secretion[6, 15]. Chloride exchanges then occur in response to shifts in these other ions. To clear an excess amount of chloride, the kidney has to prevent filtered chloride ion from following sodium back into the tubular mucosa. This can be done (a) by not reabsorbing sodium; (b) by increasing reabsorption of bicarbonate, and (c) by releasing extra ammonium, hydrogen or potassium ion into the urine, or by combinations of all three[20]. Chloride deficiencies can be compensated by reversing these processes. The extensive interaction of these mechanisms makes evaluation of any single one difficult. However, sodium and chloride form the greatest part of the renal electrolytes[10]; hence, control of chloride must depend chiefly on control of sodium. The other mechanisms to compensate selectively for discrepancies between sodium and chloride are definitely limited in capacity. Therefore, in severe differences between the concentrations of sodium and chloride, the kidney cannot compensate completely and must be assisted.

DISTURBANCES OF CHLORIDE BALANCE

In actual practice, situations where sodium and chloride are out of balance with each other are rare. Consequently, renal control of sodium coupled with chloride exchanges can compensate for most alterations. Since the two ions are associated in the diet, intake deficiencies result in depletion of salt. Similarly, in excess sweating, diarrhea and polyuria, sodium and chloride are lost together.

In vomiting or gastric suction, the material lost may be HCl rather than NaCl, and this loss of chloride without sodium will continue until serum concentrations of chloride fall[10]. This results in an excess of sodium and bicarbonate remaining behind. These two ions can be excreted in an alkaline urine (the *alkaline tide*). However, the renal bicarbonate threshold rises, and serum concentrations of bicarbonate are elevated[13]. Conversely, retention of chloride might be expected in hyperventilation when excess CO_2 loss in the lungs reduces serum bicarbonate. Concentrations of serum chloride increase as compensation for this, but protein buffer systems also come into play as compensating mechanisms, and urinary loss of chloride is only minimally increased[17]

In administration of excess chloride without sodium, as HCl or NH_4Cl, serum concentrations of the anion rise[5, 17]. The excess chloride can be removed directly by urinary excretion without sodium loss, since the cation originally given with the chloride is available for excretion by the kidney to accompany the urine chloride[17].

The thiazide and mercurial diuretics, as well as cardiotonic glycosides, induce increased output of water by the kidney by suppressing tubular reabsorption of sodium and chloride[15]. The mechanism is prevention of active transport of sodium back to the tubule from the filtrate, chloride remaining passively behind with the cation. This effect is most marked with the mercurial diuretics. With some of these, more chloride than sodium is excreted in the urine, suggesting the possibility of a primary action on some active mechanism for chloride reabsorption. The chloride excess can be explained, however, by the increased excretion of potassium which is associated with the effect of the drug. In this, sodium is exchanged for potassium, chloride again merely remaining behind to accompany the excreted potassium[15].

SUMMARY

Chloride, as other anions, is not directly subject to elaborate endocrine controls, and, for the most part, does not participate in the active transport processes of cations. Usually, the sole source of chloride intake is the food, while it is lost through sweat, feces and urine. Following absorption, it is ubiquitously distributed to all tissues, where it is largely extracellular in location. The processes of absorption, transfer and excretion are due in most areas to its passive movement along electrochemical gradients produced by active transport of cation. In the gastric mucosa, however, at least part of the chloride secretion appears to involve an active transport mechanism, and other instances of such a mechanism are noted. Chloride is also released into the gastrointestinal tract through salivary, bilary, pancreatic and upper intestinal secretions; in all of these its elaboration is largely passive, following sodium transport. Chloride is strongly absorbed in the lower intestinal tract. The major control of chloride balance is situated in the kidney. Here filtered chloride is reabsorbed passively along with sodium, or may remain in the urine along with sodium, hydrogen, potassium, ammonium or any other cation being excreted. Control of chloride by the kidney is indirect, resting largely on control of these cations. Reciprocal relationships between chloride and bicarbonate ions exist with chloride shifts in response to bicarbonate alterations, and some degree of mutual substitution in urine. In most abnormal states, chloride excess or loss is associated with similar changes in sodium.

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The Regulation of Sulfate Metabolism

JOSEPH T. BRIERRE, JR., M.D., THOMAS E. WHEELER, Ph.D., and LARRY G. DICKSON, M.D.

INTRODUCTION

Ionized sulfate (inorganic) is present in serum in small quantities compared to the majority of the electrolytes. The concentration of serum inorganic sulfate ranges from 0.3 to 1.1 mg./ml., when expressed as elemental sulfur[1]. Despite this small contribution to the total electrolyte composition, sulfate represents the end product of metabolism of sulfur in a number of biologic processes which are quite diversified. The catabolism of protein must be considered a major source of the serum sulfate; hence, one cannot divorce the discussion of inorganic sulfate from that of organic sulfate. The exclusion of one or the other would result in an incomplete discussion of the regulation of sulfate metabolism. The concentration of sulfate differs in the intracellular and extracellular fluid spaces; therefore, a determination performed on a hemolyzed specimen will not reflect the true concentration in serum[1].

SULFUR METABOLISM

Sulfur is an essential element of life. It is present in such substances as thiamine, insulin, growth hormone, glutathione, methionine, and taurocholic acid. It is absorbed by the gastrointestinal tract as inorganic salts such as Na_2SO_4, K_2SO_4 and $MgSO_4$ (Table I).

TABLE 1 REGULATION OF SULFATE METABOLISM

Source of Sulfate		
Inorganic		
	Salts:	Na_2SO_4, K_2SO_4, $MgSO_4$
	Bacteria:	H_2S
Organic		
	Non-protein:	Sulfalipids, Sulfatides
	Protein :	Amino acids (cysteine, methionine) Glycoprotein Mucin Ovomucoid Chondroitin sulfate in tendon and cartilage

Organic substances of two types serve as another source of dietary sulfur. The first is nonprotein organic sulfur, as in sulfalipids and sulfatides. The second is protein sulfur, such as that present in amino acids, glycoprotein and chondroitin sulfate of tendon and cartilage. Sulfides formed by the bacterial action of the gastrointestinal tract constitute a

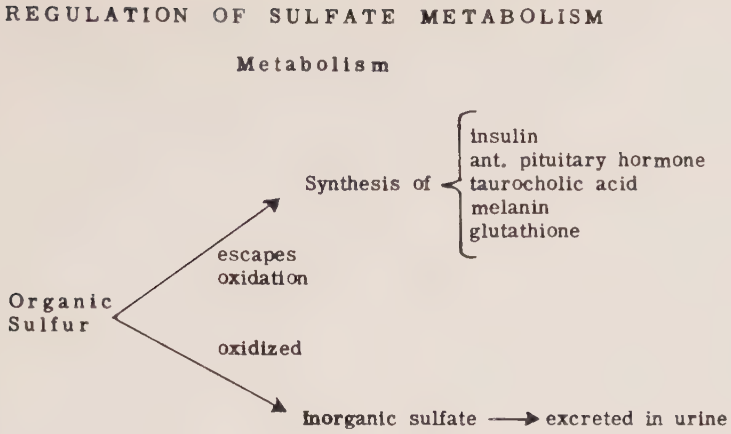

Fig. 1

minor source of sulfur. These sulfides are rapidly oxidized by the liver to form ionic sulfate. Studies performed on marine animals indicate that inorganic sulfate of sea water passes through the gastrointestinal tract with little absorption compared to that of sodium and potassium[8]. Rosenthal[12] points out that absorption of sulfate as $S^{35--}O_4$ in *Lebisthes* and *Danio* is geometric in water relatively deficient in SO_4,$^{--}$ and linear only within a certain range of concentration in the ingested water. This suggests that absorption of sulfate is a controlled process; however, the nature of the controlling mechanism is uncertain.

Once absorbed, a portion of the organic sulfur which is not oxidized is incorporated in the synthesis of insulin, growth hormone, taurocholic acid, melanin and glutathione (Figure 1). The majority is oxidized to sulfate and excreted in the urine. The remainder of the inorganic sulfur combines with various phenol derivatives, such as indole, to form ethereal conjugates which are then excreted in the urine (Figure 2).

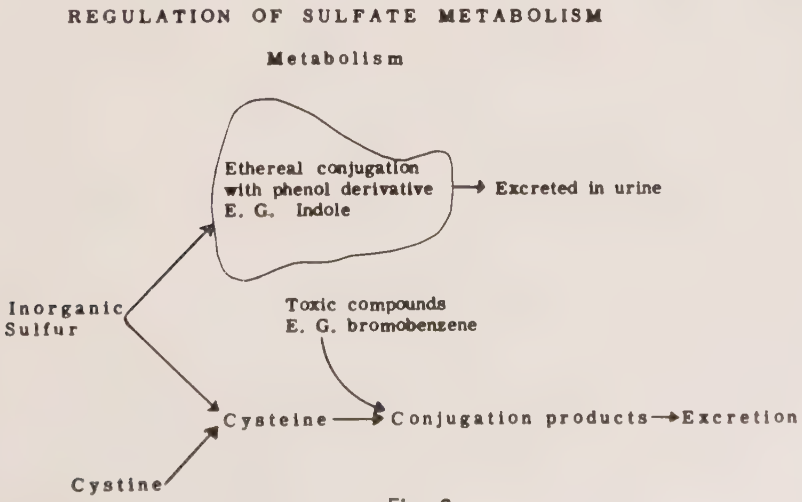
REGULATION OF SULFATE METABOLISM
Metabolism
Ethereal conjugation with phenol derivative E. G. Indole
Excreted in urine
Inorganic Sulfur
Toxic compounds E. G. bromobenzene
Cysteine
Conjugation products
Excretion
Cystine

Fig. 2

Sulfates also are conjugated for inactivation and excretion of several of the steroids, as well as estrogens. Benzene is likewise excreted as a conjugated sulfate.

EXPERIMENTAL

An analysis of several studies permits the understanding of the role of sulfate in the synthesis of more complex substances. A factor referred to as the serum *sulfation factor,* believed to be related to growth hormone activity, has been shown to stimulate the incorporation of $S^{35--}O_4$ into mouse cartilage during the formation of chondroitin sulfate[2]. Dziewiatkowski[6, 7] found $S^{35--}O_4$ to be concentrated in cartilage more than any other tissue. Costal cartilage of hypophysectomized rats incubated in human serum incorporates $S^{35--}O_4$. The rate of uptake is proportional to the log of the quantity of serum. Chesley[2] has shown that this rate of uptake is significantly reduced if the serum used is obtained from patients who have a pituitary dysfunction. In his report he utilized five patients with Sheehan's syndrome. The relationship of the substance referred to as the serum *sulfation factor* to the growth hormone of the pituitary has not been clarified.

Utilizing a similar index of activity, Salmon[13] showed that testosterone propionate administered to hypophysectomized rats markedly enhanced the uptake of $S^{35--}O_4$. When growth hormone is added to the system, there is a proportionate additive uptake of the sulfate. Further endocrine regulation has been observed by Denko[5]. Experimental exophthalmos of guinea pigs is due to an increased size of Harder's glands and to edema of the extraocular muscles and retrobulbar connective tissue[11]. Wegelius[15, 16] found that thyrotrophin stimulates the rate of uptake of sulfate in the orbital tissue of the guinea pig. He also found that thyrotrophin causes an increased uptake of SO_4^{--} in the thyroid gland, cartilage and fat. Pasternak, *et al.*[9] demonstrated an increased concentration of serum sulfate in experimentally-produced Vitamin A deficiency. Associated with this increased serum level there was an increased urinary excretion. According to Cantarow and Trumper[1], sulfur is carried in the serum and in red blood cells in several forms.

TABLE 2 REGULATION OF SULFATE METABOLISM

Transportation	
Serum:	
1. Inorganic sulfur	0.5 - 1.1 mg per 100 ml
2. Ethereal sulfate	0.1 - 1.0 mg per 100 ml
Whole Blood:	
1. Neutral sulfur	1.7 - 3.5 mg per 100 ml

These are listed in Table 2. The neutral sulfur of whole blood is derived from glutathione present in red blood cells.

The concentration of sulfate in the glomerular filtrate is approximately 1 mg. per 100 ml. and in the urine 20 mg. per 100 ml.; therefore, under normal conditions, it is partially reabsorbed by the renal tubules[3]. With a slight increase in the filtered load, the transfer mechanism is exceeded, and excess quantities appear in the urine. The majority of sulfate excreted is inorganic (Table 3). The distribution of the various sulfated compounds within the urine is dependent on diet and rate of catabolism. In chronic renal disease with decline in the filtration rate and maintenance of

TABLE 3 REGULATION OF SULFATE METABOLISM

EXCRETION	
2.5 gms daily; diet dependent	
Inorganic	85 - 95 %
Na_2SO_4	
K_2SO_4	
$CaSO_4$	
$MgSO_4$	
$(NH_4)_2SO_4$	
Organic	5 - 15 %
Ethereal conjugates	

tubular reabsorption capability, sulfate excretion is impaired, and there is a concomitant rise in the serum concentration.

WHAT IS THE CLINICAL SIGNIFICANCE OF SERUM SULFATE?

One of the philosophies behind the analysis of the serum for one of its constituents, is that a specific pathologic process will mediate some change in the physiologic condition of an individual that will be reflected by a specific change in the concentration of that constituent within the serum. This ideal condition does not exist. The level of serum sulfate is altered in many disease states. It will be elevated in any condition which obstructs the route of excretion of sulfate; indeed, the most dramatic elevations are seen in cases of renal functional impairment. Denis and Hobson[4] investigated the serum electrolytes in twenty-two patients with nephritis and cardiovascular disease. Of the ions analyzed, SO_4^{--} was the most consistently elevated. The elevated level only expresses impairment of elimination of catabolic products and does not differentiate, for example, nephritis from obstructive uropathy.

Obstructive conditions of the gastrointestinal tract with absorption of bacterial metabolic products will result in an elevated serum sulfate. Elevation is also observed in most catabolic states, a febrile condition being an example. Endocrine control has already been discussed. Vitamin dependency has been reported by several investigators[9, 10]. With so many diversified factors influencing the level of serum sulfate, knowledge of the serum sulfate level is of little clinical significance. One possible exception is chronic renal disease, where serial determinations may be of value in estimating the extent of the disease as well as the progress of the disease. Concentrations of serum sulfate from 1.5 to 3.5 mg. per 100 ml. have been observed in patients with benign prostatic hyperplasia, bladder neck contracture, glomerulonephritis, thyrotoxicosis, fever of undetermined origin, psoriasis, and arteriosclerotic heart disease. These patients had *normal* or *borderline* elevations of their blood urea nitrogen.

SUMMARY

A discussion of some of the numerous factors which influence the level of the serum sulfate has been given, with emphasis on the necessity for a thorough understanding of these factors, prior to interpretation of an absolute value of ionized sulfate. Investigations suggest that measurement of serum sulfate may be useful in evaluating borderline renal disease. For research purposes, the use of sulfate as a bioassay indicator has furthered our knowledge of complex processes such as mucopolysaccharide synthesis.

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The pH of Serum, Cerebrospinal and Intracellular Fluids in Acute Acid-Base Disturbances

DAVID M. TRAVIS, M.D.

The state of the buffer system in blood gives an incomplete story of acid-base conditions for the whole organism. Often, a useful interpretation of blood analyses requires additional information about other fluid compartments or related physiologic events.

Acid-base discrepancies between body compartments arise in several ways and may be of critical importance to physiologic response. A well-known example is potassium depletion, in which blood of the animal becomes more alkaline but muscle more acid[30]. In such an animal renal tubular cells behave as if they were also acidotic. Diuretics act in paradoxical fashion: mercurial agents, usually inactive in ordinary metabolic alkalemia, are active[19]; carbonic anhydrase inhibitors, which are normally active in the usual metabolic alkalosis, give little renal response in the K^+ depleted animal[16]. A reverse situation develops when cesium or rubidium is allowed to displace potassium from cells: acidemia develops presumably by exchange with intracellular hydrogen ions so that an alkaline reaction occurs in cells[25]. Discrepancies between blood and other fluid compartments also develop in acute acid-base disturbances. This chapter describes the relationships between blood and cerebrospinal fluid (CSF) or intracellular fluid (ICF) during onset or recovery from derangements of the $CO_2 - HCO_3^-$ system.

Opposite shifts in pH of blood and cisternal CSF occur during acute metabolic acidemia or alkalemia. Intravenous acid administration in the form of NH_4Cl produces acidemia. In spontaneously ventilating dogs, this acidemia is accompanied by some respiratory compensation and an alkaline shift in CSF as illustrated in Table I, Experiment A[28]. Winterstein suggested that NH_3 derived from infused NH_4Cl passed from blood to CSF and formed NH_4OH to give this alkaline reaction[44]. There are strong arguments against this view: (a) Stabenau, Warren and Rall[35] found that intravenous acid leads to accumulation of infused $NH_3 - NH_4^+$ in blood and not in the CSF. (b) Cestan, Sendrail and Lassalle[8] showed long ago that intravenous HCl gives the same alkaline shift in CSF without infusion of ammonia in any form; this is illustrated in

TABLE I THE pH OF ARTERIAL BLOOD AND CSF DURING ACID AND $NaHCO_3$ INFUSIONS FOR THIRTY MINUTES *

				HCO_3^-		P_{CO_2}		pH		H^+	
EXP.	I. V. SOLUTION	DOGS (n)	VENTILATION	PLASMA mM	CSF mM	PLASMA mm Hg	CSF mm Hg	PLASMA UNITS	CSF UNITS	PLASMA M x 10^{-9}	CSF M x 10^{-9}
A	Control	5	Spontaneous	22.62	22.56	44	55	7.33	7.22	47	60
	NH_4Cl	5		16.74	22.50	35	44	7.29	7.32	51	48
B	Control	2	Spontaneous	25.82	24.15	54	61	7.30	7.22	50	60
	HCl	2		18.00	24.42	46	42	7.22	7.41	60	39
C	Control	5	Constant P_{CO_2}	20.02	21.70	32	40	7.44	7.37	36	42
	NH_4Cl	5		15.82	22.27	31	39	7.33	7.39	47	41
D	Control	2	Spontaneous	24.12	23.23	50	63	7.30	7.17	50	67
	$NaHCO_3$	2		33.65	24.32	60	73	7.37	7.14	42	72
E	Control	2	Constant Vol.	17.08	22.80	22	34	7.50	7.42	31	38
	$NaHCO_3$	2		35.57	22.16	30	43	7.68	7.32	21	48
F	Control	3	Constant P_{CO_2}	19.24	22.38	26	44	7.43	7.33	37	47
	$NaHCO_3$	3		29.26	22.54	30	41	7.64	7.36	23	43

*Adapted from Robin *et al.*, 1958[28].

Table I, Experiment B[28]. (c) Robin and co-workers[28] demonstrated the critical role of carbon dioxide by holding P_{CO_2} constant during intravenous administration of NH_4Cl (Table I, Experiment C): the result was reduction of the alkaline shift in CSF in spite of aggravation of the acidemia.

Another example of opposite shifts in pH of blood and CSF is the acid reaction of CSF during alkalemia produced by intravenous administration of $NaHCO_3$[11, 12, 28]. The acid shift in CSF is small but usually present in anesthetized, spontaneously ventilating dogs (Table I, Experiment D), but can be exaggerated for demonstration by holding pulmonary ventilation constant so that P_{CO_2} rises proportionately more (Table I, Experiment E)[28]. Furthermore, the opposite changes in pH of blood and CSF during alkalemia of $NaHCO_3$ origin can be entirely eliminated by preventing a rise in P_{CO_2} (Table I, Experiment F)[28].

The failure of some investigators to demonstrate an opposite shift of pH of CSF in human subjects after $NaHCO_3$[3, 15] appears to be related to a lack of change of P_{CO_2} in CSF obtained by lumbar tap. In some instances this can be attributed to better ventilatory regulation in the unanesthetized subject[37], so that P_{CO_2} in the blood changed very little. There is also a definite lag in the equilibration of lumbar CSF with cisternal CSF or with blood[10].

These and other studies demonstrate the relatively slow transfer of HCO_3^- or H^+ between blood and CSF which accounts for the nearly exclusive dependence of CSF on changes in P_{CO_2} for any shift in pH in acute metabolic acidosis or alkalosis.

Two equations are useful in describing the principles involved. The Henderson-Hasselbalch formulation states that:

$$pH = pK' + \log \frac{HCO_3^-}{CO_2 \rightleftharpoons H_2CO_3} \quad \text{(equation I)}$$

Acute alteration of H^+ or HCO_3^- in blood is not immediately reflected in changes in HCO_3^- of CSF because of low diffusibility. The H^+ itself is also poorly diffusible across most cell membranes. Since CO_2 (or un-ionized H_2CO_3) is highly diffusible from blood into CSF, an acute alteration in blood P_{CO_2} is promptly reflected in CSF. Therefore, in these circumstances, there is an inverse relation between pH of CSF and P_{CO_2}. It should be noted that carbonic anhydrase catalyzes the hydration of CO_2 and thereby provides undissociated carbonic acid in proportion to the concentration of CO_2.

Another expression can be used to show the relationship of P_{CO_2} of the alveoli ($P_{A_{CO_2}}$) directly to CO_2 output ($\dot{V}_{CO_2}$) and inversely to alveolar ventilation ($\dot{V}_A$),

$$P_{A_{CO_2}} \sim \frac{\dot{V}_{CO_2}}{\dot{V}_A} \quad \text{(equation II)}$$

Since the P_{CO_2} of CSF is in equilibrium with jugular venous blood which in turn bears a consistent relationship to arterial (or alveolar) blood P_{CO_2}, then the combination of equations I and II allows simple statement of the sequence of events. Acid administration stimulates ventilation and lower P_{CO_2} in blood and CSF (equation II). As a result the acidemia is minimized but nearly always persists in some degree. By contrast, the pH of CSF rises (equation I). $NaHCO_3$ on the other hand gives an initial rise in CO_2 output which, at least in the anesthetized subject, is relatively greater than the simultaneous increase in ventilation: therefore CSF pH will fall. If P_{CO_2} is allowed to rise further by holding $\dot{V}_A$ constant, the fall in pH of CSF will be even greater. Finally, if P_{CO_2} is artificially held constant, the pH of CSF will not change.

Changes in the pH of blood and CSF occur in the same direction during purely respiratory acid-base alterations. Depressed respiration[8] and CO_2 inhalation[2, 11, 14, 15, 17, 18, 36] give acid changes in both compartments. Bleich, Berkman and Schwartz[2] (Table II) gave dogs 12 per cent CO_2 to breathe and at thirty minutes found elevations of P_{CO_2} equivalent in blood and CSF but a higher H^+ in CSF than blood ($p < 0.01$). This supports the view that CSF is less well-buffered against hypercapnia than blood

TABLE II THE pH OF ARTERIAL BLOOD AND CSF DURING INHALATION OF CARBON DIOXIDE FOR THIRTY MINUTES*

		HCO_3^-		P_{CO_2}		pH		H^+	
CONDITION	DOGS (n)	PLASMA mM	CSF mM	PLASMA mm Hg	CSF	PLASMA UNITS	CSF	PLASMA M x 10^{-9}	CSF
Normal	35	24.2	23.5	36	45	7.42	7.36	38	44
12% CO_2	6	30.8	25.9	80	90	7.18	7.10	67	80

*From Bleich *et al.*[2].

with its ready access to cellular mechanisms throughout the body, although conflicting evidence has been reported[36]. Another parallel change in pH of blood and CSF is the alkaline shift produced by hyperventilation. This occurs in hyperventilation induced by hypoxemia in dogs[14] or, as shown in Table III, by voluntary breathing in human subjects[10].

TABLE III THE pH OF ARTERIAL BLOOD AND CISTERNAL OR LUMBAR CSF DURING HYPERVENTILATION FOR TEN MINUTES*

		HCO_3^-		Pco_2		pH		H^-	
CONDITION	MEN (n)	PLASMA mM	CSF mM	PLASMA mm Hg	CSF	PLASMA UNITS	CSF	PLASMA $M \times 10^{-9}$	CSF
Normal	3	23.6	22.8	36	43	7.44	7.35	36	45
Hyperventilation (cisternal tap)	3	21.1	23.0	22	35	7.61	7.44	24	36
Hyperventilation (lumbar tap)	3*	21.1	22.4	22	40	7.61	7.38	24	41

* From Fisher and Christianson[10].
** For CSF HCO_3^- and PCO_2 n = 1.

The site of sampling of CSF is important. At ten minutes substantial lowering of Pco_2 and rise in pH where demonstrable in cisternal fluid, but the changes in both Pco_2 and pH were much less in lumbar fluid[15, 17, 18].

Interesting variations of these studies of acid-base disturbance have been carried out. Leusen[14] perfused the cerebral ventricles with acid, alkaline and CO_2—enriched fluid and obtained parallel and opposite changes in pH of blood. Portnoy, Thomas and Gurdjian[22] used glass electrodes to make continuous observations of pH, Na^+ and K^+ of cortical subarachnoid fluid. They found no alteration of Na^+ or K^+ in either metabolic or respiratory alterations of acid-base balance. Comparison with an electrode deep in the brain showed similar acid shifts in CSF and brain during hypercapnia whereas an alkaline shift in CSF during hypocapnia was not matched by significant pH change in brain.

It should be noted that the change in pH of CSF during acute acid-base disturbances is essentially passive and secondary to respiratory responses apparently initiated by the acid-base change in the blood. For these acute disturbances it is therefore difficult to conceive how CSF can serve as an immediate regulator of certain responses of the central nervous system as often proposed[43]. Acute acid-base responses in CSF serve as a model for events occurring elsewhere across other barriers as in cells or subcellular particles. The internal environment of the cell has considerable import for subcellular function. For example, mitochondrial oxygen uptake varies inversely with H^+ in surrounding medium; carbon dioxide, independent of any influence on environmental pH, depresses mitochondrial respiration.[39] Such responses are of course modified by other factors in the cellular environment. It can be shown that mitochondrial depression by CO_2 is dependent on the presence of calcium in the surrounding medium[38].

Intracellular pH of muscle has received attention similar to that given CSF in acute acid-base disturbances. Wallace and Hastings[41], using estimates based on CO_2-HCO_3^- concentrations, found that intracellular pH of muscle was relatively stable after intravenous HC1 or $NaHCO_3$ in spite of substantial acidemia or alkalemia. Waddell and Butler[40] and Brown and Goott[6] made similar studies using an improved method for measuring intracellular pH based on distribution of a weak acid, 5, 5-dimethyl-2, 4-oxazolidinedione (DMO) and also found relative stability of muscle pH. Consistent paradoxical shifts in pH of muscle were not observed by these workers during these metabolic disturbances. Apparently, the experimental conditions (e.g., time, anesthesia) were such that P_{CO_2} changes were small in nearly all the animals. However, in one animal receiving $NaHCO_3$, the P_{CO_2} rose 11 mm. Hg and a definite acid change in muscle was found[6]. Both groups working with the DMO method found an increase in H^+ of muscle as great or greater than that in blood during 30 per cent CO_2 inhalation and a decrease in H^+ of both compartments during hyperventilation. It would appear that acute respiratory disturbances give parallel shifts in pH of blood and muscle similar to blood and CSF. The well-established low permeability of muscle to H^+ and HCO_3^- and high permeability to CO_2 suggest that muscle pH is not only independent of blood pH[41] but probably responds paradoxically like CSF whenever experimental conditions allow spontaneous change in P_{CO_2} or exaggeration of this change by mechanical ventilation.

Brain has been studied by the DMO method[13]. As in the case of muscle and CSF, inhalation of CO_2 gave an acid shift in brain equal to or greater than that in blood (Table IV, Experiment B). The older CO_2-HCO_3^- method for estimating intracellular pH has given similar results[4, 20]. At constant pulmonary ventilation (and hence constant P_{CO_2}) intravenous HC1 gave little change in pH of brain (Table IV, Experiment C). By contrast, intravenous $NaHCO_3$ gave a slight alkaline shift in brain (Table IV, Experiment D) suggesting that brain is somewhat permeable to HCO_3^- and hence more vulner-

TABLE IV THE pH OF ARTERIAL BLOOD AND INTRACELLULAR FLUID OF BRAIN DURING CO_2 INHALATION FOR ONE HOUR AND INFUSIONS OF HC1 AND $NaHCO_3$ FOR THIRTY MINUTES*

				HCO_3^-	P_{CO_2}	pH		H^+	
EXP.	CONDITION	DOGS (n)	VENTILATION	PLASMA mM	PLASMA mm Hg	PLASMA UNITS	BRAIN UNITS	PLASMA M x 10^{-9}	BRAIN M x 10^{-9}
A	Normal	28	Constant	14.1	23	7.42	7.08	38	83
B	10% CO_2	5	Constant	17.2	83	6.90	6.69	125	205
C	HC1 i.v.	6	Constant	8.6	22	7.15	7.07	70	85
D	$NaHCO_3$ i.v.	6	Constant	35.4	29	7.73	7.18	18	66

*From Kibler *et al.*[13]. Intracellular pH of brain is calculated using the sulfate space of brain.

able to metabolic alkalosis than CSF or muscle. However, there might also be some protection against the risk of tetany during acute hyperventilation. This protection would, of course, be offset in chronic hyperventilation by diminished buffering capacity of brain[5].

Observations with DMO have been extended to intramitochondrial pH which appears to vary inversely with P_{CO_2}[27]. In tissues where intracellular pH is entirely dependent on changes in P_{CO_2}, it seems reasonable to expect that acute acid-base disurbances should give similar changes in pH of cells and their mitochondria.

The relationships of various body fluid compartments during acute acid-base alterations are of fundamental interest in a number of experimental and clinical situations. (a) Distribution of drugs during acute acid-base disturbances depends, among other things, on pH gradients between compartments of body fluid[23, 34] and must, therefore, also depend on respiratory contributions to shifts in pH across cell barriers. This contribution may vary widely with experimental or therapeutic conditions, e.g., anesthesia, mechanical ventilation, and with ventilatory status in pulmonary insufficiency. (b) A clinical picture of generalized fits, coma and multifocal stimulation of the central nervous system can occur with alkalemia brought on by mechanical over-ventilation of patients with severe respiratory insufficiency. Of five such patients recently described by Rotheram, Safar and Robin[29], two recovered when P_{CO_2} was allowed to rise. Too rapid correction of hypercapnia would appear to be dangerous because of a drastic cellular alkalosis. (c) Likewise, rapid rise in P_{CO_2}, leading as it does to an acidosis more severe in brain than blood, would be far more deleterious than gradual onset of hypercapnia over days and months. Certainly rapid induction of hypercapnia by depressant drugs or gases, airway obstruction or rebreathing of CO_2 represent serious threats. (d) During correction of hypercapnia, blood HCO_3^- sometimes fails to fall to normal, and alkalemia appears[26]. This alkalemia has been attributed to K^+ deficiency in certain patients recovering from respiratory insufficiency[24] and has been demonstrated in Cl^- deficient animals with sustained elevation in renal reabsorption of HCO_3^-[33]. (e) The mechanism for intracellular acidosis in severe K^+ depletion seems to be an exchange with H^+. However, there is a possibility that elevation of P_{CO_2}, which is often described in this condition, may contribute to the demonstrable intracellular acidosis in muscle or kidney. (f) Patients recovering from a variety of metabolic acidoses often go through a phase of hyperventilation and low P_{CO_2} long after blood pH has returned to normal[42]. Studies of pH in CSF have now provided evidence, long-lacking in such patients, that there is indeed a persistent acidosis elsewhere in the body. Correction of metabolic acidosis by $NaHCO_3$ is accompanied by persistent acidosis in CSF, or at times, aggravation[3, 31]. During correction by hemodialysis, blood pH and HCO_3^- rise toward normal. The P_{CO_2} also rises, and the CSF, therefore, becomes paradoxically acid[1, 9, 21]. (g) In chronic metabolic acidosis, the HCO_3^- and pH of CSF may be nearer normal than the blood values[1, 3, 7, 9, 15, 21, 31]. However, it should be noted that this convenient compensation does not occur in patients with chronic respiratory insufficiency in whom an approximately equivalent acid shift is found in blood and CSF[18, 32].

In *summary,* acute acid-base disturbances arising from metabolic causes can produce acidemia or alkalemia with relatively little change in pH of CSF, muscle or brain compared to change in pH of blood. Under certain conditions in which P_{CO_2} is allowed to fall (with acidemia) or rise (with alkalemia), a paradoxical shift in pH of CSF occurs. Purely respiratory alterations give parallel shifts in pH of blood and other fluid compartments such as CSF, muscle and brain. The relationships between blood and other fluid compartments are fundamental to an understanding of physiological events associated with onset and recovery in acute acid-base disturbances.

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The Regulation of Water Metabolism

A. Regulation of Osmotic Pressure

JOSEPH S. HANDLER, M.D.

This discussion of the regulation of osmotic pressure concerns normal man in whom plasma osmolality is maintained at about 280 mOsm per kg. H_2O. Some of the aberrations that occur in disease will be considered in other chapters. The regulation of the osmotic activity of plasma or serum is concerned with the regulation of the osmotic activity of all body water, since under ordinary circumstances all tissues are in osmotic equilibrium with extracellular water. The obvious exceptions to this generalization are the fluids within the gastrointestinal tract, sweat and salivary secretions, and urine, as well as some of the cells responsible for the formation or absorption of these fluids.

The physiology of regulation of osmotic activity can be considered in terms of a large reservoir of body water and solute amounting in an adult to around 40 l. with an osmotic activity of 280 mOsm per kg. H_2O. Ordinarily, daily turnover amounts to 3 to 8 per cent. The osmotic activity of this dynamic reservoir depends on the quantity as well as the osmotic activity of fluid ingested and excreted.

In this context, it is worth considering the concept of free water clearance, developed by renal physiologists to indicate net gain or loss to the organism of water in excess of solute. The estimation of free water clearance is indicated in equation I:

$$C_{H_2O} = V - \frac{U_{osm} \cdot V}{P_{osm}} \quad \text{(equation I)}$$

C_{H_2O} is the clearance of free water, and has the dimensions of volume per unit time. V is the liters of urine excreted per unit time, U_{osm} is the osmolality of the urine, and P_{osm} is the osmolality of the plasma. It is evident that C_{H_2O} is positive when urine is more dilute than plasma, and negative when the urine is more concentrated than plasma. The utility of the calculation is illustrated by considering the conservation of water affected by the excretion of a concentrated urine. Equations II and III are two examples of data that might be obtained from thirsting men over a twenty-four hour period.

$$C_{H_2O} = 0.4 - \frac{(1120)\ (0.4)}{280} = -1.2 \text{ L. per 24 hrs. (equation II)}$$

$$C_{H_2O} = 1.0 - \frac{(560)\ (1.0)}{280} = -1.0 \text{ L. per 24 hrs. (equation III)}$$

In the first example, equation II, only 400 ml. of a very concentrated urine (1120 mOsm per kg. H_2O) is excreted. The free water clearance is −1.2 l. per day, or in other words, the conservation of 1200 ml. of water free of solute. In the second example, the urine osmolality is lower (560 mOsm per kg. H_2O), the total volume of urine is higher (1 l.), and the negative free water clearance is less than in the first example, despite the excretion of somewhat more solute than in the first example. Similar calculations can be made for water and solute ingested.

Most of the information has been obtained from studies of control mechanisms in lower mammals. This is particularly true of salt and water intake since psychological factors are often of major significance in man. There is considerable evidence to support the existence of a thirst center in the central nervous system[1]. The thirst center is located in the hypothalamus. It has been suggested that this center is responsive primarily to the osmotic activity of its surrounding extracellular fluid and in a minor degree to peripheral stimuli, such as dryness of the mouth and pharynx[2]. Undoubtedly, a large number of factors, including those of psychological origin, affect drinking activity. Another aspect of the problem involves the selection of the tonicity of intake. Under certain situations, animals will ingest a salty solution in preference to a more dilute solution. However, the major stimulus here may be a deficiency of salt rather than hypotonicity of body water[3]. The recognition of satiety is also a complex function. Denton and his colleagues have demonstrated that animals will rapidly drink enough fluid to replace their deficit and then stop drinking well before the ingested fluid could have been absorbed from the gastrointestinal tract[3].

The ability of the organism to maintain a relatively constant serum osmolality despite wide variation in solute and water is finally a function of the kidney. When serum osmolality is low, a dilute urine is excreted, and when serum osmolality is high, a concentrated urine is excreted. The mechanism of control of this function was clarified by the studies of Verney and his colleagues who showed that the kidneys are responding to vasopressin, the mammalian antidiuretic hormone, and that the elaboration and release of this hormone is a function of a hypothalamic-neurohypophyseal system. It is now generally accepted that there are osmoreceptors in the hypothalamus (near the region of the thirst center), which respond to an increase in the relative osmotic activity of their environment by stimulating the neurohypophysis by way of neural pathways to secrete antidiuretic hormone[4]. The hormone, in turn, causes the kidneys to excrete a concentrated urine, thereby conserving water. In the absence of the hormone, the kidneys excrete a urine that is more dilute than plasma. The mechanism of the effect of antidiuretic hormone on the kidney has been reviewed recently[5, 6]. Suffice it to say that the hormone increases the permeability of the distal nephron to water, and that water moves passively along osmotic and chemical gradients.

This discussion is concluded with a summary of Verney's experiments in which trained dogs were the experimental subjects. One of the experiments reported in his *Croonian Lecture*[7] illustrates the rate of urine flow in response to the injection of hypertonic solutions into the arterial blood supplying the brain. Each injection consisted of 2 ml. of equi-osmolar solutions: 1.28 M. NaCl, 0.98 M. Na_2SO_4, and 2.39 M. dextrose. Each of these injections containing a solute that enters cells slowly, caused a reduction in the rate of urine flow, and an increase in the concentration of the urine. An equi-osmolar injection of 2 ml. of urea had little effect. Verney explained the latter observation by suggesting that in contrast to the first three solutes employed, urea rapidly permeated the hypothalamic osmoreceptors and therefore had little effect. The other agents remained extracellular and caused the osmoreceptors to lose water to their hypertonic surroundings and shrink, thereby stimulating the secretion of antidiuretic hormone by the neurohypophysis. He also demonstrated that the osmoreceptor system was sensitive to an increase in osmolality of as little as 2 per cent, that is about 6 mOsm per kg. H_2O. If the neurohypophysis was removed, or if the 2 ml. of hypertonic solution was injected into a peripheral vein, and thereby diluted before reaching the central nervous system, there was no change in urine flow.

Returning to these functions in man, the story is more complex. The osmoregulatory system is affected by a number of factors such as fear, pain, trauma, and certain autonomic reflexes, and is stimulated by many drugs including several general anesthetics, barbiturates, and opiates[4]. On the other hand, the inhibitory effect of alcohol on the neurohypophyseal system and the resulting water diuresis are well recognized. In addition, renal function is influenced by other hormones and drugs, as well as circulatory factors.

In *summary,* the remarkable constancy of the osmotic pressure of body fluids as sampled in serum is primarily controlled by an osmoreceptor system in the brain. When the osmotic activity of extracellular fluid rises, the center stimulates the secretion of antidiuretic hormone by the neurohypophysis. The hormone causes the kidneys to excrete a concentrated urine, conserving water for the organism. In the absence of antidiuretic hormone, the kidneys excrete a dilute urine.

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B. Abnormalties of the Secretion of Antidiuretic Hormone

DONALD P. TSCHUDY, M.D.

Abnormalities of the secretion of antidiuretic hormone involve either the failure to produce adequate amounts of the hormone or the inappropriate secretion of the hormone. The former is known as diabetes insipidus, whereas the latter syndrome occurs in association with several different disorders.

Diabetes insipidus is characterized clinically by polydipsia and polyuria. The criteria for establishing a diagnosis of true diabetes insipidus include (a) history of persistent polydipsia and polyuria, with daily urine volumes of 5 to 20 L; (b) consistently low urinary specific gravity that can not be raised above 1.010 by the withholding of fluids; (c) failure to evoke significant antidiuresis by stimuli such as hypertonic saline or nicotine, which promote the release of hormone from a functional neurohypophyseal system; and (d) antidiuresis and urine concentration following administration of 0.1 unit of vasopressin (Pitressin)[1]. A test for the ability to secrete antidiuretic hormone was devised by Hickey and Hare[2]. This consists of the intravenous administration of 0.25 ml. of 2.5 per cent NaCl per kg. body wt. per min. for forty-five minutes to individuals in whom water diuresis has been established. An antidiuretic response is seen in normals, but not in patients with diabetes insipidus. The disease is relatively rare and occurs as both an inherited and acquired form. The hereditary type occurs as a dominant disorder and accounts for about 2 per cent of all cases[1]. About one third of the acquired type have no demonstrable pathologic findings. The physiologic mechanism of the water loss seen in this disease can be readily explained by the lack of action of antidiuretic hormone on the kidney. It is thought that vasopressin acts in the distal convoluted tubule and collecting tubule to facilitate the passive back diffusion of water by altering membrane permeability. The tubular reabsorption of water in the distal and collecting tubules occurs as a passive phenomenon because an osmotic gradient has been created across these membranes whose permeability is influenced by antidiuretic hormone.

An understanding of the abnormalities of water and electrolyte metabolism seen in disorders where inappropriate secretion of antidiuretic hormone is thought to occur is obtained by an examination of the effects of administration of pitressin to normal subjects under various conditions[3–7]. In the experiments of Leaf *et al.*[3], pitressin tannate in oil was administered at a dose of one unit every twelve hours for periods of two to four days following an initial dose of 2.0 units. On the first day of pitressin administration there was a marked decrease in urine volume and increase in urinary concentration of solute. Body weight increased and serum sodium, chloride and total solutes decreased. These findings are a result of water retention and hemodilution. On the third day of pitressin administration there was a marked increase in urinary excretion of sodium and chloride. The hyponatremia observed at this stage is secondary to both hemodilution and increased urinary loss of sodium. The increased sodium loss following pitressin administration is thought to result from the depression of aldosterone secretion secondary to the expansion of extracellular fluid volume. Increased glomerular filtration rate may also play a role. Evidence for this concept is afforded by the fact that if fluid intake is sufficiently restricted during pitressin administration, sodium loss does not occur. Furthermore, sodium loss can be prevented during pitressin administration in the presence of a high fluid intake if salt retaining steroids are given. These data suggest that the loss of sodium during pitressin administration is not a direct effect of pitressin on renal reabsorption of sodium, but is secondary to water retention and expansion of extracellular fluid volume.

Abnormalities of electrolyte metabolism which are similar to those described following pitressin administration have been observed in several disorders, including bronchogenic carcinoma[8–10], disease of the central nervous system[11–13] and acute intermittent porphyria[14–15]. It is postulated that these findings result from the *inappropriate secretion* of anti-

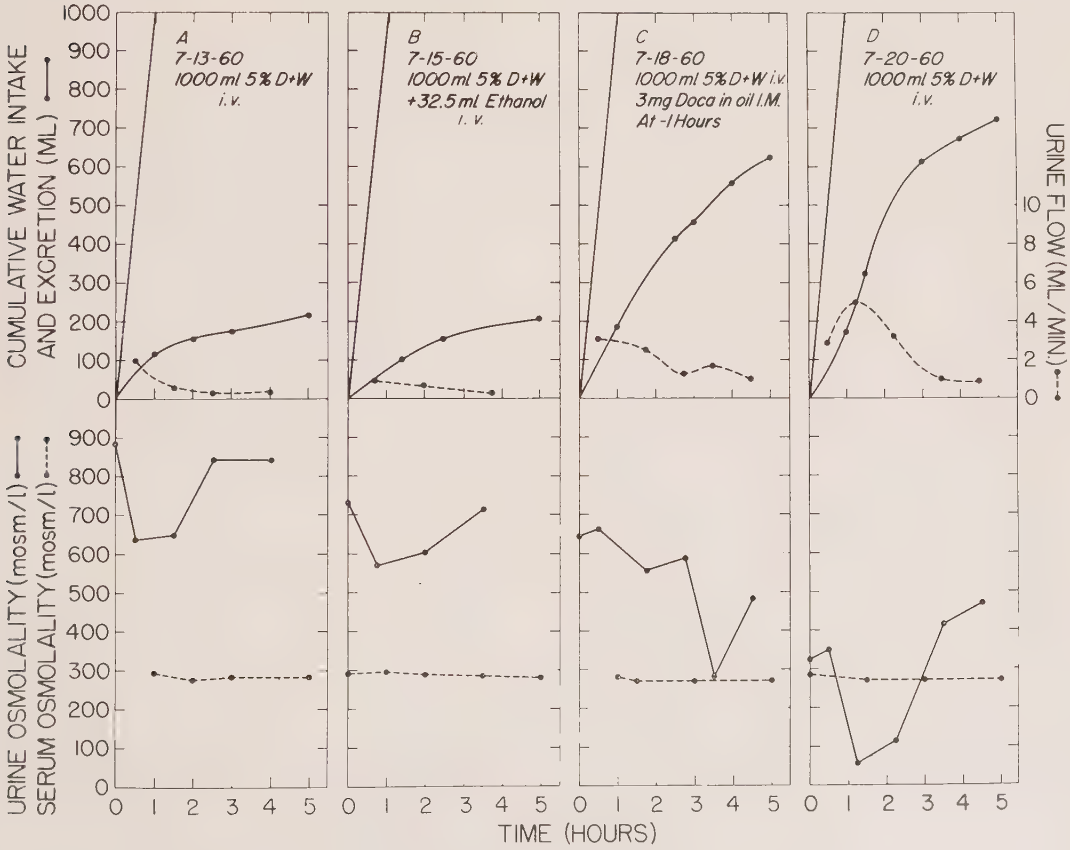

Fig. 1. Water metabolism in acute intermittent porphyria.

diuretic hormone. The hyponatremia and hypochloremia seen in tuberculosis may also result from this syndrome[16–18]. In the case of an individual with the syndrome secondary to bronchogenic carcinoma, a substance with antidiuretic hormone activity has been extracted from the tumor[19]. The mechanism leading to the inappropriate secretion of antidiuretic hormone in the other disorders is uncertain, but nervous system disease has been present in all cases.

The abnormalities of water metabolism in this syndrome are demonstrated by studies performed in a thirty-one year old woman during an attack of acute intermittent porphyria. As shown in Figure 1, four water-loading studies were performed over a period of eight days, during which the patient was rapidly recovering from her episode of porphyria. While on a constant metabolic diet 1000 ml. of 5 per cent dextrose in water was administered intravenously over a one-hour period at the start of each of the four studies as indicated by the solid line on the left of each of the upper figures. Cumulative water excretion is shown by the solid line and urine flow by the broken line in each of the upper portions of the four studies. In the lower portion of each study is plotted the urine osmolality as the solid line and serum osmolality as the broken line. On the first study (A) during the period of inappropriate secretion of antidiuretic hormone, there was a marked impairment in the ability to excrete a water-load. Urine osmolality remains very high despite the water-load. In the second study, ethanol was added to the infusion because it is known to inhibit the release of antidiuretic hormone in normal individuals. However, in the patient with the inappropriate secretion of antidiuretic hormone, again a markedly impaired ability to excrete a water load is seen with a hypertonic urine. The later studies demonstrate the increasing ability to excrete a water-load as the patient recovers from the attack of porphyria.

The recognition of this syndrome is important in that water intoxication can be prevented by the judicious administration of hypertonic saline and the restriction of fluid intake.

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Physiologic Disturbances of Fluid and Electrolytes in Diarrhea

COMMANDER G. S. HUBER

INTRODUCTION

Of all the diarrheas, perhaps the most dramatic is that of Asiatic Cholera. Instances of fluid loss exceeding 500 ml. per hr. *per anus* are not rare[17]. There is evidence that these diarrheal stools are derived entirely from an extracellular fluid space representing about 20 per cent of the patient's body weight[9]. This space includes the blood plasma whose ionic composition is presumed to represent the entire space. Since the typical cholera stool has a pH greater than 7.36 and an ionic composition of up to 149 mEq. Na per L. and 115 mEq. C1 per L. and no less than 5 mEq. K per L. and 25 mM CO_2 per L.[1], it is evident that the patient not only dehydrates rapidly but also becomes acidotic and suffers an unbalanced electrolyte depletion. For details as to the evaluation and management of the cholera patient, reference should be made to the recent literature[10, 11, 14, 15, 16].

The recent spread of this disease throughout Southeast Asia has stimulated a considerable amount of scientific investigation. The physiologic aspects of these studies have led to some interesting conclusions regarding the pathogenesis of cholera. The enormous diarrheas of cholera evidently do not arise from an increased secretion of fluids into the gastro-intestinal tract, but rather from a failure of the gut to re-absorb the large volumes of fluid that normally enter its lumen[3, 5, 7, 10, 11]. In addition a few observations[8] have been made using *in vitro* cultures of *Shigella flexneri* and *Escherichia coli* from fatal infant diarrheas, which indicate that this mechanism may not be an exclusive attribute of cholera but may find general application to fluid and electrolyte losses as they occur in other diarrheal diseases. The author has no knowledge of any other work directed along this line for diarrheas produced by other agents.

BACKGROUND

Visscher and his colleagues suggested in 1944 that relatively minor damage to the intestinal mechanism for sodium transport could lead to disastrous salt depletion[13]. Watten and his co-workers speculated that Visscher's suggestion might well apply in the case of cholera[15]. Huber and Phillips found that a substance did indeed exist in cholera stools that was injurious to the active transport mechanism for sodium in amphibian epithelia[5]. The Fuhrmans reported a similar activity which they found in a commercial preparation made from *Vibrio cholerae*[3]. Several laboratories have since reported, with a curious diversity of results, on chemical and physical properties attributable to this inhibitor of active sodium transport[2, 4, 6]. Thus, one must conclude that the nature of this substance is still uncertain. In spite of the uncertainty as to the composition of this substance, evidence has been obtained that a causal relationship does exist between it and the severity of the disease[7].

DISCUSSION

A comparison of the diarrheal rates, V_x, and the *Inhibitor* excretion rates RINH, as assayed on stools using the short circuited frog skin preparation of Ussing and Zerahn[12] is seen in Figure 1. RINH is expressed as Frog Skin Units per hour, (FSU per hr). A Frog Skin Unit is defined as that amount of inhibitor which will suppress the bioelectric parameters of a short-circuited frog skin 1 per cent below the extrapolated control values. Since the inhibitor curve is parallel to and ahead of phase of the diarrhea curve, it is inferred that the diarrheal severity is dependent on the rate of production of inhibitor by the causative organism.

If consideration is now to be given to concentrations of stool cations as they relate to diarrheal severity, early and late in the course of the disease (Figure 2), a basis of formulation can be visualized. As the severity of the diarrhea increases, the concentrations of stool cations approach their respective plasma levels. The sodium values are lower and

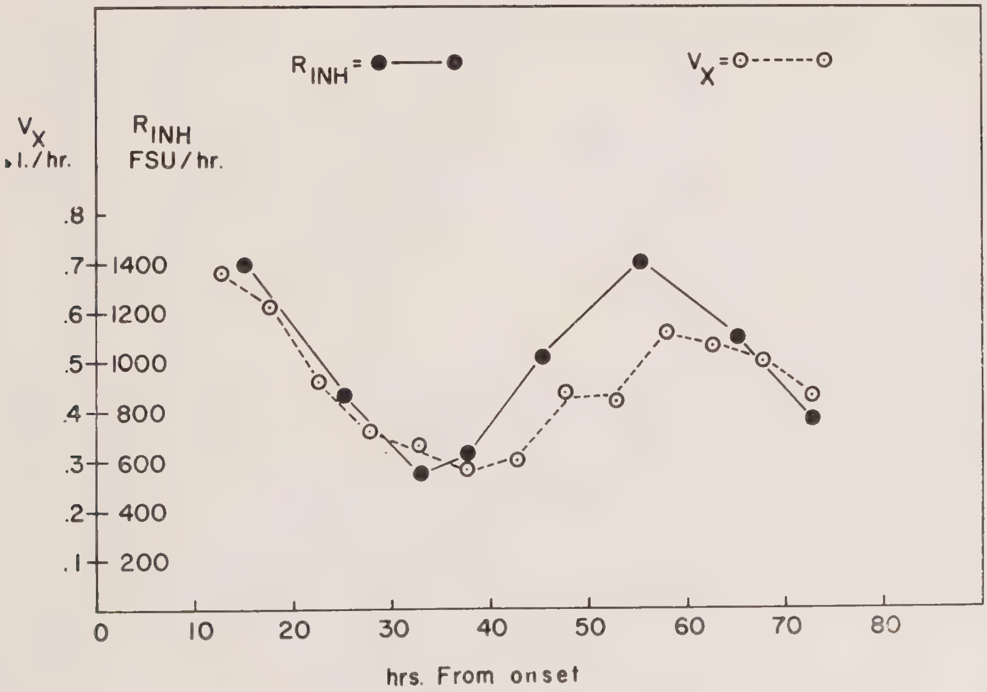


Fig. 1. A comparison of the excretion rates of diarrheal stools (V_x) and sodium transport inhibitor (R_{INH}) as they vary during the first seventy hours of cholera.

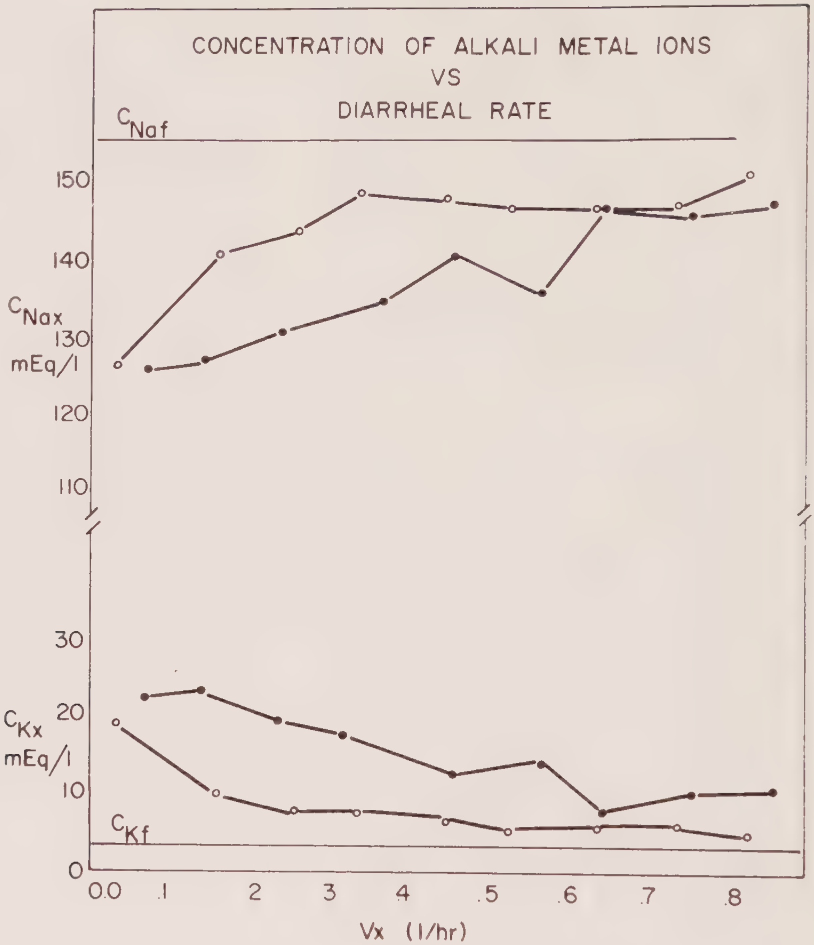
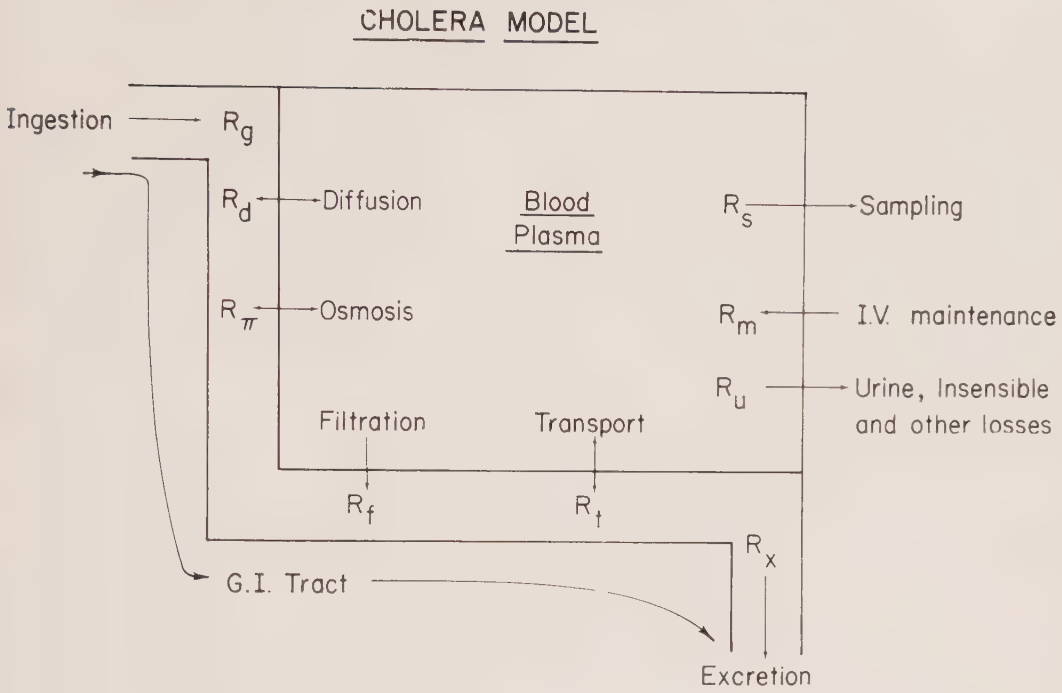


Fig. 2. Comparative concentrations of the alkali metal ions in stool and plasma as they vary with diarrheal severity early (closed points) and late (open points) in the course of cholera.

the potassium values higher in stool than their respective plasma values. Watten *et al.* made the same observation on their Thai patients[17]. This result would be expected if sodium and potassium entered the gut as plasma ultrafiltrates but only sodium were removed (at a higher concentration) by active transport. Increases in intestinal permeability, with the attendant reduced resistance to diffusion, should account for the differences between the cases seen early and those seen late in the disease, i.e., before or after the fortieth hour from onset of diarrhea.

A two-compartment model was con-



$$R_x = R_g + R_d + R_\pi + R_f + R_t$$

$$\left(\text{Plasma} \longrightarrow \text{Gut} = (+),\ \text{Gut} \longrightarrow \text{Plasma} = (-)\right)$$

Fig. 3. A two-compartment model for cholera. The R_j are distinguished by the force that drives them across the pertinent boundary. The flux direction: plasma to gut is taken as positive. A detailed development of the equations together with their evaluation is being presented elsewhere (8).

structed, as in Figure 3, in which the fluxes of components were distinguished according to their particular driving forces. The mathematical treatment was confined to the methods of algebra for the sake of simplicity. That greater precision should be obtained with more refined mathematical treatment is admitted. Also, it should be noted that this model does not discriminate as to the anatomic location of any of the given fluxes, except that they occur somewhere along the G.I. tract across a more or less permeable boundary.

When these equations were evaluated[8] in terms of the concentrations of stool

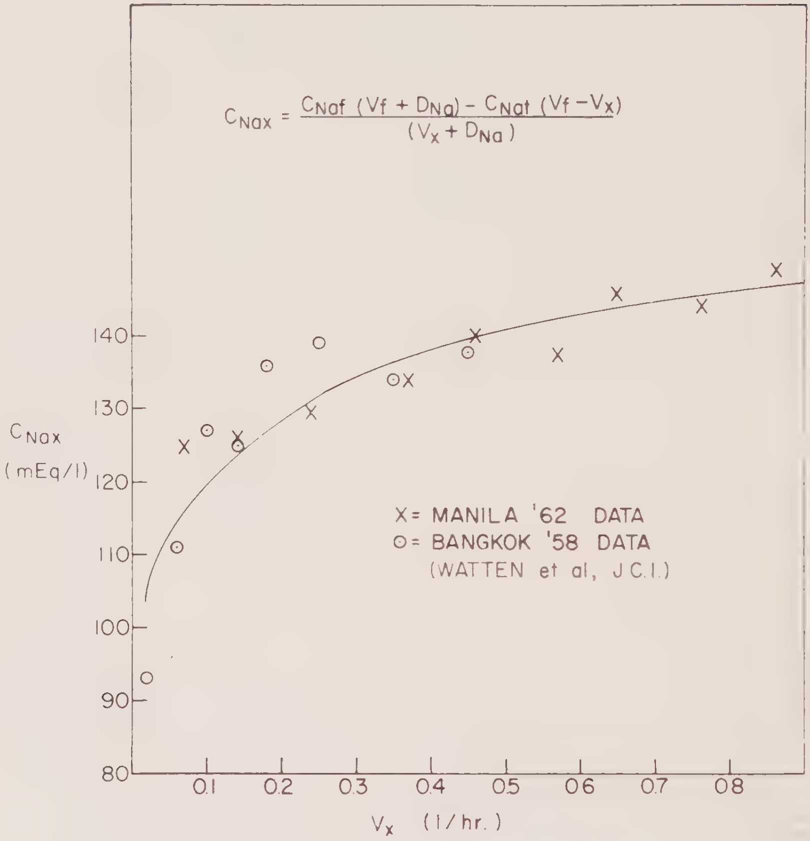


Fig. 4. Depicts the goodness of fit with experimental averages when the concentration of sodium in the excreta is evaluated at various diarrheal rates for an average cholera victim seen early in his disease.

cations to be expected at various diarrheal rates, it was seen, as in Figures 4, 5, and 6, that the results agreed well with experimentally determined values, not only for the averages of several cases but also in individual cases.

CONCLUSIONS

If the formulation is valid, one should not expect to see any drastic changes in the concentrations of serum cations in this type of diarrhea, even though the patient is suffering from severe, unbalanced depletion of cations. The sodium concentration should increase gradually in the untreated patient to an upper limit of about 162 mEq. per L. of serum water, the concentration at which sodium appears to be transported. The potassium values, on the other hand, should decrease only gradually

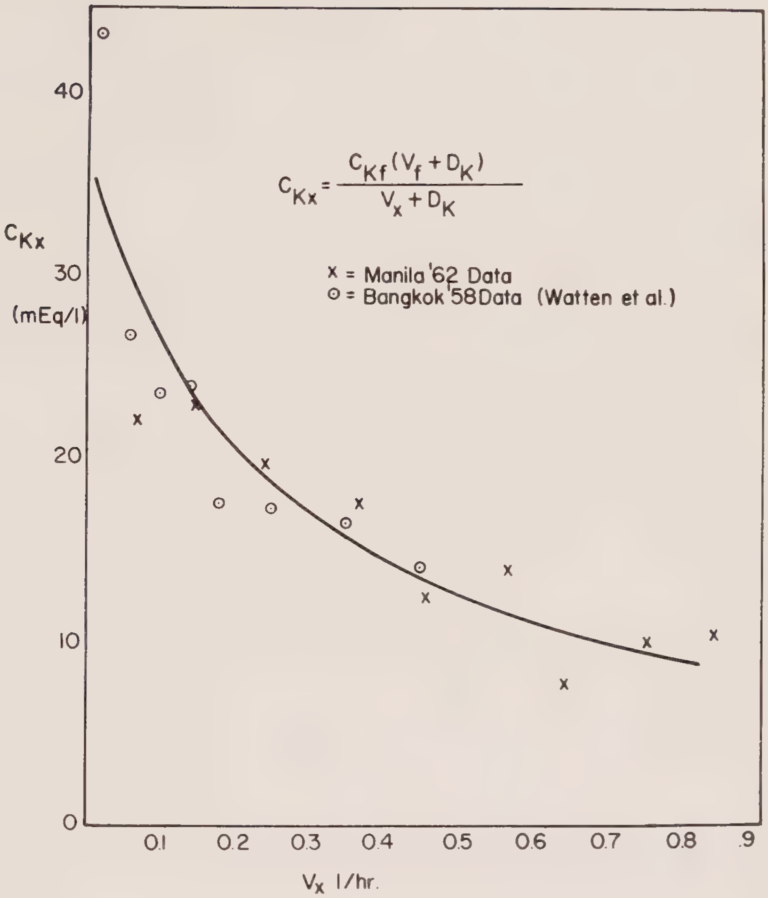


Fig. 5. Depicts the goodness of fit with experimental averages when the concentration of potassium in the stool is evaluated at various diarrheal rates for an average cholera victim seen early in the disease.

or not at all, since the potassium losses via the stools tend to be replaced from the relatively large reserves of intracellular potassium.

No cases have been studied in which patients had not received replacement therapy prior to study. Despite this, values seen early in the disease, compared to those seen late, tend to confirm the hypotheses. The average of the *early* values for serum sodium was 154 ± 5 mEq. per L. as opposed to *late* values of 156 ± 5 mEq. per L. The corresponding potassium values were 3.70 ± 1.2 mEq. per L. and 3.06 ± 1.1 mEq. per L[8]. In this connection, Watten *et al.*[15] stated that the values for serum sodium obtained on admission were elevated.

SUMMARY

Evidence was presented that the pathogenetic mechanism for the losses

Fig. 6. Depicts the goodness of fit when the concentration of sodium in the stool was calculated for each case of proven cholera reported by Watten, et al (15).

Individual Values of $C_{Na\,x}$
(calc'd from data of Watten et. al.*)

Pt #	C_{Nax} (mEq/l)		Pt #	C_{Nax} (mEq/l)	
	calc'd	obs		calc'd	obs
1	133	130	12	141	132
2	150	165	14	128	129
3	143	142	15	145	142
5	142	152	20	126	123
6	134	135	21	121	122
7	135	139	23	84	82
8	140	139	24	122	122
9	130	131	25	87	85
10	123	119			

$\sigma = \pm 5.5$

*J. Clin. Invest, 38: 1879-89 (1959)

of fluid and cations in cholera involves inhibition of the active transport of sodium. It was suggested that this mechanism might also pertain to other types of diarrhea. Algebraic treatment of a two-compartment model that included this consideration lead to results that were in agreement with experimentally determined values in cholera.

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Physiologic Regulation of Electrolytes in Cardiac Failure

MAURICE B. BURG, M.D.

Congestive heart failure is characterized by an increase in the volume of the extracellular fluid. In the absence of complications, however, there are no important alterations in the concentrations of sodium, potassium, and chloride in the serum. It is only in patients with advanced disease or following intensive therapy that there may be marked alterations in the concentrations of these ions. In this chapter, special consideration will be given to one of these states, namely hyponatremia, both because of its clinical significance and because several aspects of electrolyte physiology are involved in considering its pathogenesis[1–5].

Basic to an understanding of hyponatremia is the fact that the concentration of sodium in the serum is an index of the effective osmotic pressure of the body fluids, and not of the total sodium content of the body. In this context effective osmotic pressure is defined as that exerted by solutes which do not penetrate cell membranes rapidly, and thus are able to affect the distribution of water. Sodium salts constitute most of the effective osmolality in the extracellular fluid. Although urea contributes to the total osmotic pressure of the serum, it does not affect the distribution of water between cells and surrounding fluids, since it penetrates cells freely, and its concentration in cell and extracellular fluid water is approximately equal. In this sense, serum sodium concentration is a more significant index of effective osmolality than is the freezing point depression, since the latter measurement is affected by the freely-penetrating substances, such as urea, which are not physiologically pertinent.

There are times when serum sodium concentration and effective osmolality are not synonymous. The examples usually given are hyperglycemia and hyperlipemia. In the first instance, the presence of a relatively non-penetrating, osmotically-active substance, glucose, in the extracellular fluid increases its osmotic pressure. Mannitol, the therapeutic use of which is increasing, would have a similar effect. As regards hyperlipemia, the large lipid molecules decrease the water concentration of serum, so that the amount of Na in a volume of serum is decreased, although the physiologically significant variable, concentration in serum water, is unchanged.

It is now well established that osmotic pressure is uniform throughout the body fluids, both intracellular and extracellular. Acute alterations in the osmotic pressure of one compartment are attended by flow of water through the cell membranes, rapidly producing equality at a new level of osmolality. Thus, one may consider that the osmolality of the intracellular fluid equals that of the extracellular fluid. Further, the effective osmolality of all of the body fluids may be directly deduced from the concentration of serum sodium.

Just as sodium and its anions account for most of the osmotic activity in extracellular fluid, potassium and its anions account for most of the osmolality of the intracellular fluid. Therefore, it is not surprising that the concentration of serum Na is a relatively simple function of the total body Na, K, and water:

$$\text{Serum sodium concentration} \cong \frac{(\text{exchangeable sodium} + \text{exchangeable potassium})}{\text{total body water}}$$

Experimental proof for this has been provided by studying the relationship between the concentration of serum sodium, exchangeable body sodium, exchangeable body potassium, and total body water, both in normals and in patients with abnormalities of serum sodium[3].

Consideration of the equation suggests that either decreased total body sodium, or potassium or increased body water could cause hyponatremia. The first possibility may be excluded, since total body sodium is elevated in congestive failure, regardless of the sodium concentration in serum. The second possibility, loss of body potassium, has been demonstrated in many patients with hyponatremia and may well contribute to the hyponatremia. However, the relative importance of this mechanism is uncertain because of the difficulty in interpreting studies of exchangeable potassium. Attempts to correct hyponatremia by potassium administration have been relatively ineffective as well as dangerous. It is the third possibility, a relative increase in body water, that accounts for hyponatremia in most edematous cardiac patients. This increase in body water is, in turn, due to inability of the kidneys to excrete water normally. Thus, a consideration of the renal mechanisms for water excretion is pertinent.

Through micropuncture studies, it is well established that dilution of the urine normally occurs only in the distal portion of the nephron, that is the ascending limb of Henle's loop, the distal convoluted tubule and the collecting ducts. Fluid leaving the proximal tubule has been reduced in volume by reabsorption of water, but remains isotonic to serum. It is in the ascending limb of Henle's loop that the concentration of the tubular fluid is initially reduced below that of plasma by the reabsorption of sodium chloride in excess of water. The amount of dilute urine that can be formed and, in turn, the amount of water that can be excreted is limited by the volume that is delivered to the ascending limb. If this volume is low, as when glomerular filtration rate is reduced or excessive amounts of fluid are reabsorbed in the proximal tubule, then water excretion is limited. Reduced distal delivery of tubular fluid is one important cause of water retention associated with hyponatremia in congestive failure.

In the absence of antidiuretic hormone *(ADH)*, the dilute fluid which leaves the ascending limb is further diluted by sodium chloride reabsorption in excess of water reabsorption in the distal convoluted tubule and collecting duct, and the resulting dilute urine is excreted. When *ADH* is present, however, the permeability of the distal nephron to water is greatly increased, and water flows out readily until the tubule fluid becomes isotonic with its surroundings and an isotonic or concentrated urine is excreted. In many instances, failure to correct the dilution of the body fluid present in hyponatremia is due to the inappropriate secretion of *ADH*, and this is a second important cause of hyponatremia in the presence of congestive heart failure.

There are, then, two categories of renal abnormalities which limit water excretion in these patients: (a) diminished volume of fluid delivered to the distal diluting site, and (b) inappropriate secretion of *ADH*. Attempts have been made to determine which condition predominates in a given patient and to analyze the etiology of the abnormality. A major methodological problem has been difficulty in determining circulating levels of antidiuretic hormone. For this reason, most of the studies to date have relied on indirect tests of renal function.

One such test is the administration of poorly reabsorbed solutes such as mannitol[1]. It has been reasoned that these substances will reduce the fractional reabsorption in the proximal nephron, delivering a greater volume to the distal where urinary dilution takes place. There are a number of patients with hyponatremia and congestive failure who respond to infusion of mannitol with an increased flow of dilute urine and increased concentration of serum sodium. In these patients, excretion of potassium and acid is also enhanced, suggesting that distal tubule secretion of K^+ and H^+ had also been limited by the decreased distal delivery of sodium salts. This is in accord with the theory that potassium and hydrogen ion secretion are in exchange for sodium reabsorbed at the same distal site. Patients in congestive failure with hyponatremia and edema often benefit from effective diuretic therapy. Rather than causing a further decrease in serum sodium, as might be feared, the diuretics actually cause sodium concentration to increase. While it is true that total body sodium is decreased, the excreted urine is dilute, and relatively more water than sodium is eliminated[2]. Thus, the defect in water excretion is corrected by increased distal delivery of sodium salts allowing the kidneys to rid the body of excess water.

In the absence of practical assays for *ADH*, abnormalities in *ADH* secretion have usually been deduced from the response or lack of response to a water-load, which further dilutes the body fluids and decreases sodium concentration in serum[5]. Normally a decrease in the effective osmotic pressure of the extracellular fluid would stimulate osmoreceptors in the region of the anterior hypothalamus to reduce the secretion of *ADH* from the posterior pituitary. Why *ADH* secretion should continue in patients with hyponatremia in whom plasma osmolality is already reduced is not at all clear, nor, for that matter, is it known why thirst and water ingestion continue. Since almost any manipulation and a variety of drugs may result in antidiuresis in man, there are multiple possibilities for inappropriate or non-osmotically induced release of *ADH* in these chronically ill patients. Further, it has been suggested that the same stimulus that is responsible for salt retention may be intensified in the hyponatremic individual and that in the absence of salt ingestion, thirst and *ADH* secretion result. A possible mechanism is that because of cardiac insufficiency there is a diminution in *effective* circulatory volume despite the increased extracellular volume. Such patients with continuous, inappropriate *ADH* secretion have been identified by failure to dilute their urine despite administration of solute and water loads.

Other cardiac patients with hyponatremia, who are less severely ill, respond to water-loads by diluting their urine. Their control of *ADH* secretion is clearly abnormal since they continue to secrete *ADH* despite abnormally low levels of serum osmolality. However, when osmolality is reduced further, a diuresis ensues indicating suppression of *ADH* secretion. It is as if the control mechanism is intact, but *reset* so that it responds at a lower absolute level of plasma osmolality. The explanation for this is not clear, but may be related to the nature of the normal stimulus to the osmoreceptors. The stimulus is thought to be swelling of the pertinent hypothalamic cells due to a shift of water into them from the diluted extracellular fluid. Resetting of the osmoreceptor could conceivably be provided by a primary loss of intracellular solute.

The diminution in the content of osmotically-active substances in the cells is accompanied by loss of water, shrinkage of the osmoreceptors and stimulation of *ADH* secretion. Subsequent water retention induced by *ADH* dilutes the extracellular fluid. Water re-enters the cells until the volume of the osmoreceptors is once again normal, albeit at a lower, effective osmotic pressure, and *ADH* secretion ceases. The new equilibrium is characterized by chronic expansion and dilution of the extracellular fluid, but a normal intracellular volume. The pituitary system operates precisely as in the normal, responding to rises and falls in effective osmotic pressure, although at a lower level. In essence, the lowered concentration of serum sodium in these patients is viewed as being *normal* for them, and both the renal capabilities, insofar as dilution is concerned, and the activity of the hypothalamic-neurohypophyseal system are undisturbed.

The therapy of chronic dilutional hyponatremia, even when physiologically sound is often unsuccessful. Water restriction will ultimately increase the sodium concentration of the serum, but the resultant thirst makes the procedure a difficult one to pursue. Osmotic diuretics, insofar as they promote the excretion of water in excess of sodium, will temporarily alleviate the hyponatremia, but the manipulation is difficult, requiring large amounts of the osmotic diuretic and may, on occasion, be hazardous. Diuretics in responsive patients, as indicated earlier, may also promote the excretion of water in excess of sodium and reverse the hyponatremia. Of itself, a diminution in serum osmolality is not a serious problem, unless it is permitted to progress to the point where symptoms of water intoxication develop. The most sensible approach to therapy would appear to be reasonable limitation of water intake and focusing of attention on any measures, particularly the judicious use of diuretic agents, which might improve the underlying congestive failure[4].

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Serum Electrolytes in Inanition, Fever and Hypothermia

F. WILLIAM SUNDERMAN, M.D., Ph.D.

In the normal process of living, the electrolytes in the body are supplied with the food. When food is withheld and salts are not otherwise provided, the electrolyte reserve in the body must be utilized. In this connection, it should be emphasized that sodium is not a natural constituent of vegetable foods and is contained in only limited quantities in animal foods. Furthermore, considerable amounts of sodium stored in the body fluids may be lost by sweating and excretions. In contrast, potassium is a constant component of most foods so that any individual who receives or absorbs food is unlikely to develop a deficiency of potassium. Bearing these physiologic considerations in mind, it seems desirable and of interest to examine the changes in electrolyte pattern and fluid distribution under fasting conditions. Moreover, it is recognized that disease states are closely allied to various stages of inanition and, therefore, the changes observed during fasting are useful in interpreting the changes observed in disease.

A number of years ago, opportunity was afforded for the intensive study of a severely emaciated man who had voluntarily undergone a continuous fast for forty-five days[7]. His reasons for fasting were based upon a religious asceticism. Insofar as we are aware, this person's fast was of the longest duration for which scientific measurements were obtained. Our findings in this individual (Bernard) (Fig. 1) have been compared to those obtained in a patient with anorexia nervosa[11] (Fig. 2) who might be regarded as suffering from a slow or chronic starvation of more than two years' duration.

Since metabolic processes, as with all chemical reactions, are influenced by the temperature of the reactants, it is desirable to consider the effect of body temperature on the electrolytes and distribution of the body fluids. Considerable data have been accumulated concerning the disturbed electrolyte patterns in the serum of patients during the febrile period of pneumonia[4, 5, 9]. From data obtained in past years, it is possible to construct a composite electrolyte pattern typical of that occurring during the fever of lobar pneumonia.

Opportunity has also been afforded to study the serum electrolytes in a child with hypothermia associated with the invasion of the hypothalamus by a hemangioma[10]. Rectal temperatures taken over a period of four weeks ranged from 90° to 94° F., with occasional temperatures as low as 88° F.

In Figure 3 are portrayed the normal electrolyte pattern, the patterns in the fasting subject and in the patient with anorexia, a composite pattern for lobar pneumonia, and the pattern observed in the child with hypothermia[13].

ELECTROLYTE PATTERNS DURING ACUTE AND CHRONIC STARVATION

It will be seen in figure 3 that in both the fasting man and the patient with anorexia nervosa, the concentrations of serum total base and chloride were decreased. In the fasting subject, the concentration of serum chloride on the forty-fifth day of fasting was 74 mEq. per liter, and in the patient with anorexia nervosa, values of serum chloride between 50 and 70 mEq. per liter were practically always obtained. Although this patient vomited after meals on a number of occasions and especially when chlorides were administered intravenously, nevertheless, it would seem that the decreased concentration of serum chloride cannot be accounted for on this basis. Moreover, a similar decrease in chloride was observed in the

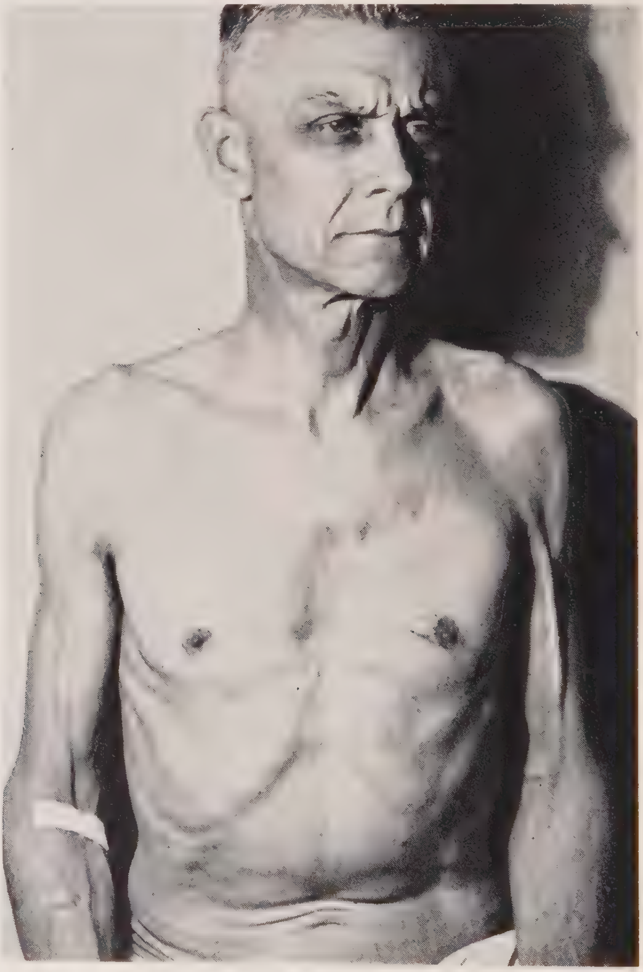

Fig. 1. Photographs of Bernard five days after discontinuing his fast and after he had gained sixteen pounds in weight (Amer. J. Clin. Path. 17:170, 1947).

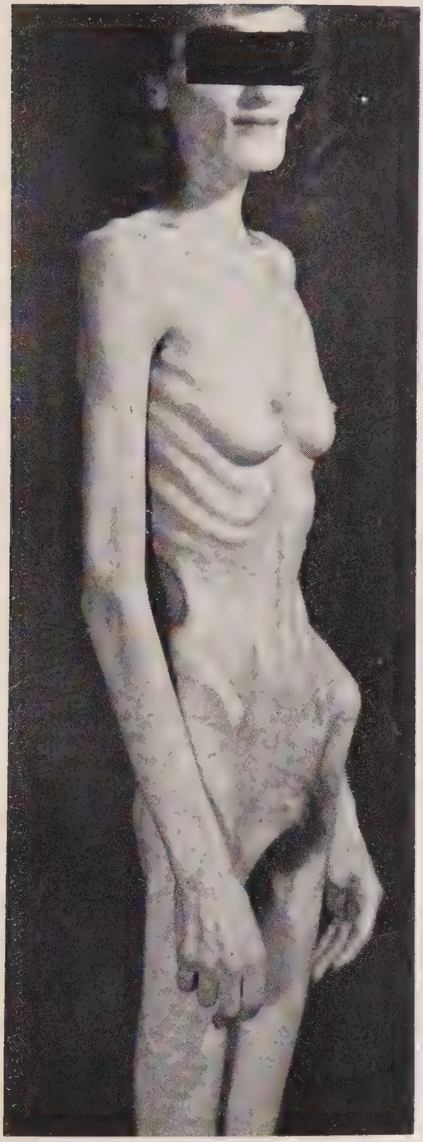

Fig. 2. Patient suffering from anorexia nervosa (J. Clin. Endocrinol. 8:210, 1948).

fasted individual who had not vomited at any time during his fasting period.

It will also be seen that the decreased concentration of serum chloride, in both of these individuals, was compensated for by an increase in the concentration of bicarbonate. Although this relationship is unexplained, it seems reasonable to relate it to the diminished intake of chloride with substitution of bicarbonate derived from endogenous sources.

The observed decrease in the concentrations of serum total base obviously represents diminished concentrations of serum sodium. The diminutions in concentrations of serum chloride in both of these patients were much greater than the diminutions in serum total base. In the case of Bernard (prolonged fasting), the diminution in chloride amounted to approximately 27 mEq. per liter and that of total base, 5 or

6 mEq. per liter; in the patient with anorexia nervosa, the diminution in chloride amounted to more than 40 mEq. per liter, at a time when diminution in total base amounted to only 17 mEq. per liter. Thus, it would seem that the factors regulating the serum electrolytes during fasting and anorexia nervosa appear to be more directly concerned with maintenance of concentrations of total base closer to that of the normal than in the distribution of the individual anions.

At the end of the fasting period, the concentration of calcium in Bernard was increased. Although the reason for the increased concentration is not evident, it is possible that this may be a reflection of the loss in skeletal weight during fasting and, presumably, release of the calcium of bone. The increased concentrations of magnesium observed in the serum of both persons has been of particular interest. Hypermagnesemia is a characteristic finding in hibernating animals and was also observed in the hypothermic child.

ELECTROLYTE PATTERNS IN FEVER AND HYPOTHERMIA

It will be seen that both in fever and hypothermia there is decreased concen-

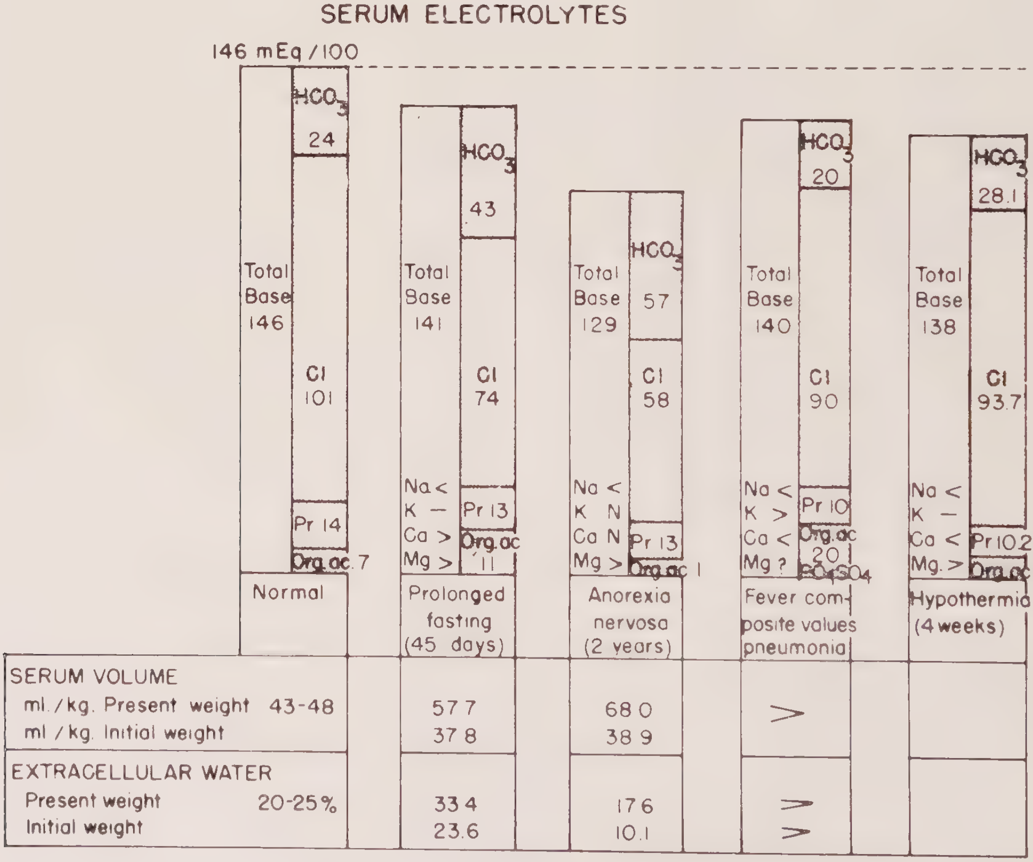

Fig. 3. Serum electrolyte pattern observed in fasting, anorexia nervosa, fever and hypothermia (Amer. J. Clin. Path. 21:325, 1951).

tration of total base with corresponding decrease in serum chloride. The bicarbonate concentration is deceased in the serum of febrile patients. This is consistent with the findings of Stadie, Robinson and Austin[2] that, either at constant CO_2 tension or at constant pH, an elevation of temperature lowers the concentration of CO_2. Conversely, an increase in the concentration of CO_2 was to be expected in the serum of the child with hypothermia.

In marked febrile states, such as pneumonia, the appetite and the power of digesting food become so impaired that it is difficult to give the patient more than 800 to 1000 calories per day, although the individual's metabolism may require an many as 4000 or more calories to supply the body requirements. The combined inadequacy of food and the destruction of tissues from the infection lead to an extremely high breakdown of the patient's own body proteins so that with a nitrogen intake of 7 gm. or less per day, the nitrogen excretion may be 30 gm. or more, corresponding to about 700 gm. of tissue catabolized per day. The electrolytes that are released when tissue cells are catabolized are especially potassium, sulfates, phosphates and, under conditions of incomplete oxidation, organic acids. The increase in the concentrations of these components in the serum reflects to a conspicuous degree the excessive and rapid tissue catabolism during infections.

The most spectacular change in the electrolyte metabolism in febrile conditions, such as pneumonia, is the diminution in the excretion of chloride and total base in the urine with corresponding decrease in their concentrations in serum. These disturbances appear to be at least in part due to the same influences that are observed in the fasting subjects, namely, inanition and diminished ingestion of salt.

In both fasting subjects (Fig. 3), the values for serum volume were greatly increased when calculated in relation to the actual body weight. However, when calculated in relation to the initial or ideal weight, the values for serum volume were decreased below the normal range. The difference in the volume of extracellular fluid between Bernard and the patient with anorexia nervosa is striking. In Bernard, the extracellular fluid volume was elevated in relation to his fasting weight but was within the normal range of values when calculated in relation to his prefasting weight. On the other hand, the patient with anorexia nervosa revealed a striking percentile decrease in extracellular fluid volume in relation to her actual weight and obviously in relation to her ideal weight. It seems possible, therefore, that the dissimilarity in percentage of extracellular water may be due to differences in the lengths of time during which the conditions lasted.

All the available evidence points to a retention of body fluids during fever similar to that observed during inanition. For example, the patient's loss of weight during the febrile period of pneumonia is much less than would be expected, considering the catabolism and inadequate food intake. Indeed, there may be an actual gain in weight during pneumonia[4] owing to retention of water. This may amount to 500 to 1000 gm. per day, for several days in succession. After the febrile period diuresis may occur for a period of from one to three days.

SUMMARY

A brief resume has been presented of the electrolyte patterns in the serum during acute and chronic starvation and during fever and hypothermia. Disease states are closely allied to various stages of inanition and fever so that the changes observed during fasting and in relation to the temperature of the body are useful in interpreting the changes observed during disease.

Since large reserves of water and electrolytes are contained in both the extracellular and intracellular fluids, sudden changes in the concentration of the serum electrolytes are usually averted. It is only when the loss of water and electrolytes occurs without restitution or where excessive amounts of electrolytes are given, that marked changes in the serum result. The decrease in the total base concentration is usually dependent upon a loss of cations in the urine or vomitus, or through dilution of the serum. When base is needed to neutralize acids that have accumulated in pathologic states, the bicarbonate anion is the one most readily displaced, with chloride also assisting when the load becomes heavy. Bicarbonate and chloride thus assume a secondary role in the electrolyte pattern of serum. The factors regulating the serum electrolytes appear to be more directly concerned with the maintenance of a constancy of total base than with a normal distribution of bicarbonate and chloride.

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Alterations in Electrolytes in Renal Disease

A. The Serum Electrolytes in Renal Insufficiency

F. WILLIAM SUNDERMAN, M.D., Ph.D.

Renal insufficiency may result from a variety of disorders and may produce an equal variety of electrolyte disturbances[1, 2, 3]. The character of such disturbances depends in large measure upon the severity of the renal damage, as well as on extrarenal factors, such as edema, dehydration, infection, anemia, etc., which may complicate the picture.

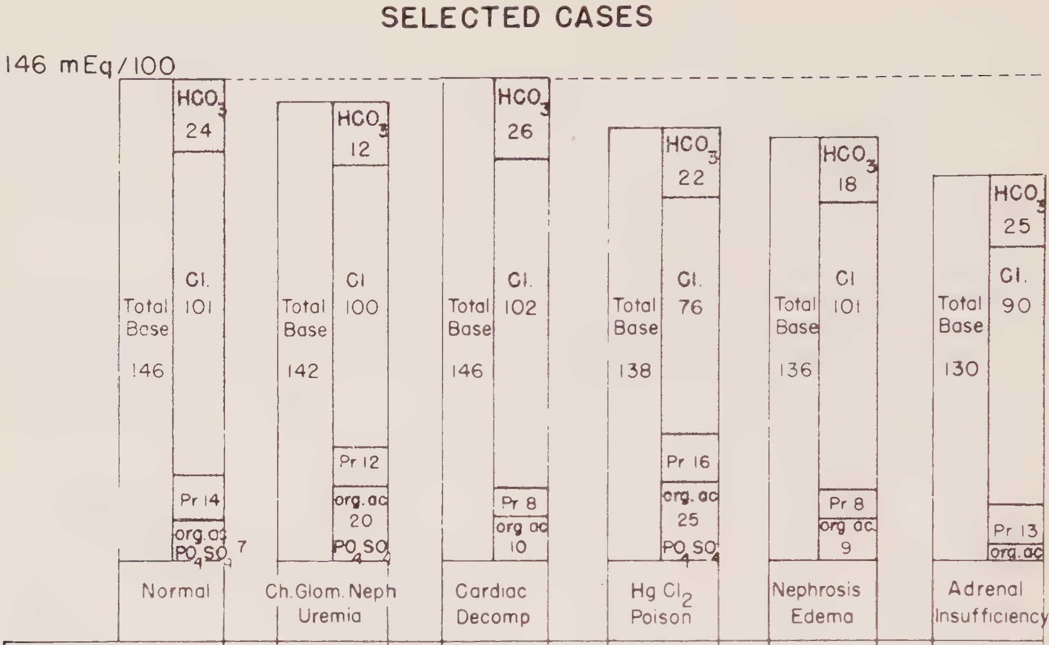
It should be recognized that the components that make up the body media are in a constant state of flux. While the concentrations of the components may be different in the various media of the body, nevertheless, for each medium, they are maintained in a state of relative stability through the balancing of a number of physicochemical forces. In the maintenance of this constancy, the kidney plays a major role by the simultaneous performance of several functions which include (a) assistance in the regulation of the osmotic pressure, (b) the excretion of end products of metabolism, and (c) the synthesis of certain components such as ammonia and hippuric acid.

It is almost impossible to correlate morphologic changes in the kidney with chemical patterns found in the serum, and no attempt will be made at this time to do so. This chapter is concerned primarily with observations of the serum electrolytes in individuals suffering with various stages of renal insufficiency in the acute, chronic, and terminal stages.

ELECTROLYTE CHANGES IN ACUTE AND CHRONIC RENAL INSUFFICIENCY

In Figure 1, an attempt is made to portray some of the electrolyte disturbances observed in acute and chronic renal insufficiency. Selection and classification of patients are sometimes difficult. The patterns given in this figure were observed in fairly typical cases.

In the patient with chronic glomerulonephritis and uremia (Fig. 1, column 2), it will be seen that the concentration of total base is essentially within the normal range of values and that the principal disturbance in the electrolyte pattern consists of diminished bicarbonate concentration with compensatory



	Normal	Ch.Glom.Neph Uremia	Cardiac Decomp	$HgCl_2$ Poison	Nephrosis Edema	Adrenal Insufficiency
NPN-mg./100	30	227	40	282	26	36
CHOLESTEROL	180	192	172	—	546	273
FREEZING PT.°C	-.56	-.65	-.54	—	—	—

Fig. 1

increase in the phosphate, sulphate, and organic acids. Most uremic patients show this type of acidosis. In some patients, particularly if vomiting occurs, the chloride concentration may also be decreased. The kidney also conserves the loss of base in urine through the synthesis of ammonia and the replacement of sodium by ammonia. In the patient with uremia, there was a marked increase in the concentration of non-protein nitrogen and an increase in the depression of freezing-point. In our experience, when the depression of freezing-point of serum becomes as great as 0.65 C., the prognosis is ominous.

In the patient suffering with renal insufficiency (Fig. 1, column 3), as an accompaniment of cardiac decompensation, the disturbance in the electrolyte pattern is observed chiefly in the decreased concentration of base bound by protein, which is compensated by a slight increase in the concentrations of phosphate, sulphate, and organic acids. This patient, with the pattern shown in the figure, had edema. There is little doubt that the most responsible factor in the production of edema is the depletion of protein owing to the increased permeability of the kidneys to serum albumin. It would appear in some cases that the loss of protein in the urine is insufficient to account for the diminution in serum protein. As a consequence, it is frequently postulated that a disturbance in the manufacture of protein also occurs. It will be seen that in the patient with cardiac decompensation there is some elevation of non-protein nitrogen with decreasing concentration of serum cholesterol. In our experience, increasing non-protein nitrogen and decreasing cholesterol concentration is attended by a poor prognosis.

The patient poisoned by bichloride of mercury (Fig. 1, column 4) had anuria for six days and almost continuous vomiting. A considerable portion of the decrease in chloride can be accounted for by the increase of phosphate, sulphate, and organic acids. The relative rise in these anions with displacement of chloride is probably attributable to the loss of the selective excretion through the kidneys of phosphate, sulphate, and organic acids which are constantly being liberated in metabolism. That such selective excretion normally occurs is indicated by the fact that the ratio of the normal daily excretion of phosphate, sulphate and organic acids in the urine to the daily chloride excretion is many times greater than the ratio of the normal concentration of phosphate, sulphate, and organic acids in the serum to the concentration of chloride in the serum. This patient's pattern illustrates the readiness with which chloride may be reduced in the serum to make way for other anions.

In the patient with lipoidal nephrosis (Fig. 1, column 5), there is a marked decrease in total base without similar decrease in concentration of chloride. In other words, their seems to be a preferential loss of sodium from the serum. Preferential chloride retention is one of the most striking and important findings in nephrosis. That this patient was edematous is evidenced by the diminution in the base bound by protein. In such patients, the judicious use of sodium lactate appears to be helpful in restoring the normal electrolyte pattern, especially if the plasma proteins are also replaced. The reasons for the disturbance in fat metabolism in these patients are as yet unknown, but the disturbance appears to be related in some way to the hypoproteinemia.

Impairment of renal function is usually observed in patients with adrenal cortical insufficiency. The essential electrolyte disturbance is a decreased concentration of sodium and chloride. In the patient whose electrolyte pattern is given in Figure 1, column 6, the urea clearance was decreased to 23 per cent of average normal function. Impairment of renal function in adrenal cortical insufficiency appears to be the result of adrenal failure alone, since coincidental kidney lesions are usually not found histologically.

Thus, consideration and comparison of the electrolyte patterns of serum may afford valuable information regarding the type of renal impairment that may be encountered and may aid in treatment as well as in prognosis.

TERMINAL RENAL INSUFFICIENCY

To determine the role played by the electrolytes and nonelectrolytes in advanced renal insufficiency, measurements were made on the sera of selected patients in the terminal stages of uremia. The analyses were made on blood samples obtained just before or immediately after death. An effort was made to select either those patients who had died before replacement fluids were administered or those in whom it was believed that the replacement therapy did not influence significantly the chemical pattern of the serum. A period of several

TABLE I. COMPOSITION OF SERUM TERMINALLY

Case	mEq. per liter								mg. per 100 ml.							t‡	Diagnosis
	Total Base	Potassium	Calcium	Magnesium	Chlorine	Bicarbonate*	Protein†	Phosphorus**	Phosphorus	Non-protein Nitrogen	Urea Nitrogen	Uric Acid	Creatinine	Glucose	Cholesterol	Degrees C	
									UREMIA—PREDOMINANTLY RENAL (GROUP A)								
1 (L. H.)	131	6.6	4.3	2.7	74.8	15.7	13.5	7	12.3	202	154	19.5	17.8	135	219	0.585	Malignant hypertension
2 (J. E.)	132	7.8	4.5	2.0	84.6	6.3	12.8	7	12.1	220	190	11.7	12.9	118	255	0.596	Malignant hypertension; chronic glomerular nephritis
3 (G. E.)	135	4.2	3.5	2.3	87.8	14.3	10.0	5	9.6	...	122	9.1	15.4	154	208	0.578	Malignant hypertension; anuria convulsions
4 (G. A.)	131	4.3	4.9	2.1	83.6	11.2	8.8	11	19.5	354	228	11.8	16.8	131	313	0.667	Malignant hypertension; anuria; terminal nephritis
5 (L. H.)	144	5.2	3.8	1.9	93.9	23.3	11.1	6	10.8	216	168	9.4	13.9	120	238	0.653	Chronic nephritis; myocardial failure
6 (J. D.)	150	9.7	5.1	3.1	97.6	12.5	11.1	3	4.6	232	178	19.3	19.7	113	175	0.669	Chronic nephritis; myocardial failure; hypertension
7 (M. J.)	146	9.5	5.4	3.4	103.5	11.2	13.1	6	10.7	170	146	16.3	6.5	95	191	0.651	Chronic glomerular nephritis; coronary sclerosis
8 (P. E.)	142	5.8	3.9	2.5	87.8	18.0	10.9	7	12.1	223	166	11.5	10.1	154	192	0.648	Chronic glomerular nephritis; cerebral hemorrhage
9 (L. L.)	142	9.2	3.5	2.8	85.0	5.4	12.6	11	19.5	274	232	13.8	17.9	110	189	0.634	Chronic nephritis; tetany
10 (P. A.)	153	12.7	4.8	2.0	81.5	5.4	13.7	5	8.2	187	148	11.4	14.9	179	221	0.656	Chronic glomerular nephritis (heart blood)
11 (S. T.)	157	6.0	3.9	4.0	96.1	16.5	13.5	4	7.4	209	188	23.9	18.2	123	182	0.698	Chronic glomerular nephritis
12 (B. R.)	164	4.9	5.5	2.2	106.2	9.0	14.6	4	7.2	140	128	4.1	9.4	59	269	0.663	Chronic nephritis; dehydration
									UREMIA—PREDOMINANTLY EXTRARENAL (GROUP B)								
13 (R. O.)	136	4.3	3.9	3.2	85.8	24.6	11.6	4	7.0	...	73	8.4	10.8	123	272	0.512	Lobar pneumonia
14 (S. S.)	137	4.2	4.3	2.0	90.5	24.6	12.0	2	3.5	...	30	5.7	1.6	86	140	0.529	Delirium tremens; alcoholism
15 (P. L.)	139	3.5	3.9	1.8	92.2	23.5	10.7	2	3.6	...	51	1.3	1.8	57	185	0.526	Cirrhosis of liver
16 (S. K.)	133	8.4	4.9	2.9	72.0	30.0	8.8	6	10.1	142	168	15.1	13.7	130	171	0.562	Carcinoma of the prostate
17 (B. W.)	154	8.5	..	..	..	13.7	14.3	..	...	...	103	30.0	8.0	392	...	0.699	Diabetic coma
18 (O. B.)	144	8.1	6.6	3.3	95.2	3.6	16.7	7	12.3	245	188	18.4	16.0	180	...	0.690	Multiple myeloma (heart blood)

*$[HCO_3]$ mEq. is taken as equal to $[CO_2]$ mM. · 1.27. ** [P] mEq. is calculated at pH 7.31 where $\frac{B_2HPO_4}{BH_2PO_4} = \frac{77}{23}$ (PO_4).

†[P] mEq. = 2.14 × (Pr) Gm. per 100 ml. ‡ Freezing-point measurements were made according to the method of Stadie and Sunderman[13].

years was required to collect the cases included in this study.

The data are presented in Table I. The observations have been divided into two groups: Group A = those of patients in whom the uremia was regarded as predominantly renal in character, and Group B = those in whom the uremia was believed to be predominantly extrarenal. This classification of cases has been difficult and, to a degree, uncertain.

The first four cases of Group A all suffered from malignant hypertension. In the sera of these four patients, the concentrations of total base and chloride were markedly decreased. All of these patients gave a history of vomiting so that the low levels of these components may be attributed, at least in part, to losses from this source. When diminution of total base occurs in the absence of vomiting, the decrease is usually believed to be due to a loss of the ability of the kidneys to conserve fixed base. Since anuria was present in cases G. E. and G.A., the low concentrations observed in the sera of these two patients cannot be due to a lack of the kidney's ability to conserve cations. It might be noted that the concentrations of base bound by protein in both of these patients were low. This might suggest that the diminution of the total electrolytes was due primarily to dilution, perhaps as a result of the ingestion of non-saline fluids. Deficit of total base is essentially a deficit in sodium. This is one of the most common of metabolic disturbances observed clinically.

It might be noted that in one of these patients (G. E.) the concentration of serum calcium was diminished to 3.5 mEq. per L., at which level the patient developed convulsions. Muscular twitchings, with or without convulsions, in uremia are believed by many investigators to be caused by a decreased concentration of the ionizable calcium fraction of serum following the retention of phosphate by the kidney. Although the concentration of serum calcium at which symptoms occur is by no means fixed, nevertheless, increased muscular irritability is usually observed when the concentration of total calcium is less than 3.5 mEq. per L.

All of the measured non-protein nitrogen components in the sera in Group A patients were increased. Particular attention is directed to the increase of these components in relation to the observed freezing-point depressions. In all except the first three cases, the freezing-point depressions were greater than 0.60° C. In these cases, the concentrations of the total electrolytes were greatly decreased, and it would thus appear that the diminutions in electrolytes were being compensated for osmotically by the increase in the non-protein nitrogen components. In case 4, the total electrolytes were also markedly decreased, although the freezing-point depression was 0.667° C. It should be noted, however, that the concentration of non-protein nitrogen in the serum of this patient was increased to more than ten times normal.

Except for case 5, the bicarbonate concentrations in the sera of Group A patients were all diminished, and in all of them, with the possible exception of case 6, the phosphate concentrations were increased. These findings are typical of the acidosis observed in nephritis, in which there is retention of phosphates, sulfates, and organic acids with compensatory diminution in bicarbonate and sometimes chloride.

In cases 5 to 7, inclusive, the renal insufficiency was accompanied by cardiac decompensation. Terminally, the concentrations of base bound by protein were not decreased more than those in the sera of the other patients in this group. In renal insufficiency with myocardial failure and edema, there is usually observed a decreased concentration of base bound by protein with an increased concentration of residual anions that include lactic acid. In cases 5 to 7, the concentrations of total base were essentially within the normal range, although it should be noted that the concentrations of serum potassium and serum magnesium in cases 6 to 7 were greatly elevated. Since potassium and magnesium are prominent components of cells, the increased concentrations of these components may be related to increased cellular catabolism.

The concentrations of serum total base were elevated in the last three cases (10 to 12, inclusive,) of Group A. In only one of these, case 12, was the increase in total base accompanied by a corresponding increase in chloride. The retention of chloride in this case was accompanied by a pronounced decrease in bicarbonate, resulting in a pattern frequently referred to as "chloride acidosis."

In all but three sera in Group A, increases in the serum glucose were observed terminally. The values of serum cholesterol were variable; however, in only one serum (case 4) was there a noteworthy increase. In five out of the twelve sera, the cholesterol concentrations were below 200 mg. per 100 ml. As previously noted, when the values for nonprotein nitrogen increase at the same time that values for cholesterol diminish, the prognosis is poor.

In Table I are also given measurements of sera in Group B patients in whom the uremia was considered to be predominantly extrarenal. It is questionable as to whether all of the six cases listed should be placed in this category. For example, B. W. (case 17), in which the uremia was a complication of diabetic coma, was shown to have had extensive renal damage at autopsy. In O.B. (case 18), with multiple myeloma, the azotemia might be, at least in part, attributable to a blockage of the renal tubules by precipitated proteoses and proteins. The freezing-point depressions in both sera (cases 17 and 18) were greatly increased; in case 17 the hyperglycemia obviously contributed to this elevation.

In the other four patients in Group B (cases 13 to 16, inclusive,) the concentrations of serum total base were decreased. In case 13, the decrease in concentrations of the electrolytes, the increase in urea nitrogen, and decrease in the freezing-point depression are typical of the changes that occur during the precritical period of lobar pneumonia[4]. Slight increases in non-protein nitrogen have long been observed during the course of this infection. Cohn reporting elevations in 87 per cent of fifty-five cases that he studied[5]. It is seldom, however, that as high a level of urea nitrogen is reached as that observed in case 13. Similar types of changes are observed in the sera of patients suffering from adrenal insufficiency. In such patients, it is believed that the changes are due to failure of the adrenals, since the presence of coincidental renal disease can usually be excluded[6]. Recently, we have obtained freezing-point depressions as low as 0.49 C. in patients with acute adrenal cortical insufficiency. In our experience, a decrease in the freezing-point depression below 0.50 C. is attended by a poor prognosis.

Urea and certain of the other identified constituents of blood non-protein nitrogen are in the main non-toxic in the concentrations found in uremia; therefore, it would be difficult to hold them *per se* responsible for the symptoms that are encountered. Increase in the concentration of these components does, however, increase the total osmotic pressure, unless compensatory adjustments are made in the serum electrolytes. One of the most inherent tendencies of the organism is the maintenance of total osmotic pressure in the body fluids within a narrow range of variation. Toward this maintenance, small diminutions in the total electrolytes are compensated for by increases in the concentrations of nonelectrolytes. Since one of the most important functions of the kidney is concerned with the regulation of water and electrolyte metabolism, it follows that the regulation of osmotic pressure is also one of its important activities. In renal insufficiency, the kidney has apparently lost is ability to excrete adequately the accumulated end-products of nitrogen metabolism, as well as certain of the electrolyte components, such as phosphates, sulfates, and organic acids. As a result of such retentions, the total osmotic pressure of the serum may be increased to levels where the protoplasmic structure of the organism cannot withstand the strain. When critical levels are reached, death ensues.

DISCUSSION

It has been suggested by a number of workers that for the maintenance of normal osmotic pressure, changes in the osmolar concentration of the electrolytes balance opposite changes in the osmolar concentration of the nonelectrolytes. Thus, Herrick and Ni demonstrated an inverse relationship between chloride and sugar in whole blood[7,8]. In our laboratory, similar inverse relationships were observed in the sera of diabetic patients after the ingestion of carbohydrates and after the administration of insulin[9, 10, 11]. It is also noteworthy that in the sera of patients with chronic nephritis, the decrease in the concentrations of chloride is usually compensated by an increase in the concentration of non-protein nitrogen components[12]. Despite such compensatory efforts, the osmotic pressure of the serum in chronic nephritis is almost always increased. In a series of over one hundred measurements of freezing-point depressions in the sera of nephritics, no patient survived whose serum yielded a value greater than 0.65 C.

SUMMARY

Measurements of electrolytes and non-electrolytes have been made in the sera obtained from patients in acute and chronic renal insufficiency and in the terminal stages of uremia. These data were considered in relation to the total osmotic pressures as determined by freezing-point. Our studies support the view that in renal insufficiency with uremia, one of the most important functions of the kidney has been lost, namely, the maintenance of normal osmotic pressure relationships. It appears that the critical upper levels of total osmotic pressure, beyond which changes in the human protoplasmic structure are irreversible, correspond to freezing-point depressions between 0.65 and 0.70 C.

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B. Electrolyte Metabolism in Renal Disease Insufficiency

IRA N. TUBLIN, M.D.

SODIUM METABOLISM IN RENAL DISEASE

Abnormalities of sodium metabolism are the most commonly seen alterations in diffuse renal diseases. The inability of the chronically diseased kidney to conserve sodium is a hallmark of functional renal impairment. When pushed to extreme degrees, the tendency of the nephritic patient to lose sodium through the kidney results in salt-losing nephritis[1]. The lesser more common degree of sodium loss is often inapparent since the sodium content of the normal diet (approximately 10 gm. of sodium chloride per day[2]) exceeds the obligatory sodium loss of most nephritic patients. Renal loss of sodium thus becomes manifest when a spontaneous or iatrogenic event occurs which restricts the patient's free access to sodium.

Attempts to treat the hypertensive patient, who frequently has renal insufficiency, with a low sodium diet may lead to the depletion of extracellular sodium, hyponatremia, and an aggravation of renal insufficiency.

A similar but less obvious clinical situation ensues when the patient with chronic renal disease develops superimposed congestive heart failure. A low sodium intake is desirable when edema is present. Once cardiac compensation is achieved, care must be taken to increase the sodium intake lest the patient's renal disease increases in severity. The resulting range between too much sodium for the heart and too little sodium for the kidney is at times narrow and requires careful measurements of body weight, serum sodium, and sodium excretions to maintain the patient in a physiologic state.

The problem of sodium wasting through the urine is compounded by the common uremic symptoms of nausea, vomiting, and diarrhea which cause loss of sodium through the gastrointestinal tract. Loss of sodium, from any source, results in a decrease in extracellular fluid and a diminution in the already compromised glomerular filtration rate of the nephritic patient. A vicious spiral of advancing azotemia may result.

Other clinical settings for sodium wasting occur in acute renal disease. The diuretic phase of acute tubular necrosis[3] and the diuresis observed after the relief of lower urinary tract obstruction[4] are sometimes attended by marked depletion of sodium.

The serum concentration of sodium is a variable, dependent on the total exchangeable sodium, the total exchangeable potassium and total body water.[5] Loss of sodium, therefore, may or may not be reflected by hyponatremia.

Abnormalities in the concentration of serum sodium in the anuric patient often reflect inadvertent or futile therapeutic maneuvers by the patient or physician. Hyponatremia in the anuric patient without documented extra-renal loss of sodium most often results from water-loading in an attempt to induce diuresis. A rising weight, and a history of the administration of fluids to an oliguric or anuric patient aids in interpreting the observed hyponatremia as a consequence of dilution. The presence of edema in a hyponatremic patient is *a priori* evidence of dilution as the cause of the hyponatremia. Dilutional hyponatremia can be severe enough to cause symptoms of water intoxication which mimic the neurologic symptoms of uremia[6]. Hyponatremia can result also from the intracellular shift of sodium in the seriously ill anuric patient[7].

In unusual circumstances, hypernatremia can occur in the oliguric patient. The use of cation exchange resin of the sodium cycle for the treatment of hyperkalemia may lead to hypernatremia[8]. Water loss through the lungs may be of extreme magnitude at times and can result in significant shrinkage of total body water leading to a rise in sodium concentration[9].

POTASSIUM

Clinically, significant potassium loss in the urine is unusual in patients with chronic renal disease unless the patient is treated with drugs which induce a kaluresis. Most instances of potassium-losing nephritis have been patients with primary or secondary hyperaldosteronism or patients with renal tubular acidosis or the Fanconi syndrome[10]. Hypokalemia when seen in chronic renal disease may also be due to potassium loss through the gastrointestinal tract.

Hyperkalemia is a frequent but not consistant finding in acutely or chronically oliguric patients who have urinary outputs of less than 400 ml. per day. It is a common cause of death in the anuric patient.

The chronically azotemic patient without oliguria does not usually have marked hyperkalemia (serum potassium of greater than 7 mEq. per liter[11]). Marked hyperkalemia in such a patient can usually be ascribed to an extraordinary potassium intake through dietary or medicinal routes, the release of large amounts of intracellular potassium, or acidosis[12].

CHLORIDE

Abnormalities of the serum chlorides in renal disease are usually considered to be secondary to changes in other cations and anions[13]. However, the electrolyte pattern of hyperchloremic acidosis is a relatively specific one which occurs in a limited number of patients with renal disease.

Renal tubular acidosis is a rare, acquired, or congenital renal disease in which there is an inability of the kidney to excrete acid urine. The primary cause of the syndrome is unknown. The tubular defect is accompanied by a normal or near normal glomerular filtration rate and therefore, "tubular insufficiency without glomerular insufficiency"[14] exists. In the absence of retention of sulfate or phosphate (which depend on glomerular filtration rate for excretion), the systemic acidosis is accompanied by hyperchloremia[15].

A more common type of hyperchloremic acidosis is seen in chronic renal disease with a decrease in glomerular filtration rate and with significant azotemia. This pattern of severe hyperchloremic acidosis occurs at relatively low levels of blood urea nitrogen and is most frequently[16] but by no means exclusively, seen in patients with chronic pyelonephritis. The hyperchloremia usually decreases spontaneously as the blood urea nitrogen increases. This serum electrolyte pattern is also a not infrequent complication of ureterosigmoidostomy. The pathogenesis of hyperchloremic acidosis in pyelonephritis is unknown but presumptively is due to a selective pathologic involvement of the tubules. There is not, however, a defect in the ability of the kidney to produce an acid urine.

PHOSPHATE AND SULFATE

The inorganic sulfate and phosphate ions depend on glomerular filtration for their excretion. In general, their retention with concomitant elevation of serum levels are correlated with the degree of decrease of glomerular filtration rate. Of the two, serum sulfate elevation correlates best with the degree of acidosis[17]. There are no symptoms in the nephritic patient that are attributed directly to the elevation of these anions.

CALCIUM

In renal diseases, the variability of calcium absorption, secondary parathyroid hyperplasia, and severe acidosis may result in serum calcium levels that range between slight hypercalcemia and hypocalcemia. Serum calcium is usually low in uremic patients.

Severe hypercalcemia in the presence

of uremia is seen in patients with primary hypercalcemic diseases. The uremic syndrome may reverse itself when the primary disease is treated.

MAGNESIUM

Serum magnesium levels in renal disease are maintained in the normal range until a severe decrease of glomerular filtration rate occurs (less than 30 ml. per minute). The capacity to excrete magnesium in patients with renal impairment is decreased. Recently it has been pointed out that magnesium intoxication may occur through the use of antacid treatment[18].

SUMMARY

The electrolyte abnormalities associated with renal disease can be grouped into four categories:

1. Abnormalities resulting from the loss of water and electrolytes through the diseased kidney.
2. Abnormalities resulting from the retention of water and electrolytes because of the diseased kidney.
3. Abnormalities inherently a part of specific renal diseases.
4. Modifications of the first three groups secondary to treatment of the patient or superimposed extra-renal disease.

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C. Renal Lesions Associated with Electrolyte Imbalance

GONZALO E. APONTE, M.D.

Although many histopathologic changes can occur in the human kidney in association with abnormalities of serum electrolytes, few are known to be caused specifically by any given deficiency or excess. This is not surprising, since the alterations in serum electrolytes seen clinically are usually multiple, and frequently are accompanied by abnormalities of other tissue components. Furthermore, they may be the result rather than the cause of pathologic changes in the kidneys. Experimental work has helped somewhat to elucidate this problem. The variation in the susceptibility of the kidneys to changes in serum electrolytes noted among different animal species does not negate the fundamental value of these investigations. What does cast doubt upon some of the experimental work published is the poorly controlled manner in which the particular electrolyte abnormality was induced and sustained.

POTASSIUM

Hyperkalemia. It is not known whether hyperkalemia *per se* produces distinctive pathologic changes in the human kidney. Sustained increases in the serum levels of potassium are usually the result of renal disease or adrenocortical insufficiency, conditions in which it is not possible to evaluate properly the effects of hyperkalemia alone. When the hyperkalemia is secondary to disorders such as hemolytic disease and extensive body burns, the picture is complicated by associated factors such as hypoxia. However, marked excesses of any electrolyte will injure living tissue, and the kidney is not a likely exception. A probable example of the toxic effects of high potassium concentrations on cells is the recent report about the occurrence of ulceration of the small intestine in eleven patients who had been taking enteric-coated capsules of potassium chloride[7].

Hypokalemia. Cytoplasmic vacuolation of renal tubules is not a rare lesion in human disease. The vacuoles which develop as a result of potassium depletion are not stained with fat or glycogen stains. Similar ones can be seen in cases of poisoning with dioxane or diethylene glycol and following the administration of sucrose, mannitol[4] or disodium ethylenediaminetetracetic acid[80]. One of the first reports to associate the occurrence of vacuolar nephropathy with protracted diarrhea in man was that of Jaffé and Sternberg[88] in 1919. Sporadic descriptions of the lesion were published in subsequent years, but its association with intestinal disease was not firmly established until the report by Kulka, Pearson and Robbins in 1950[95]. These authors found a distinctive type of renal tubular vacuolation in eight of seventy-two patients with ulcerative colitis and in seven of eighty patients with intestinal disease of various types. All patients had had diarrhea; malnutrition and electrolyte disturbance had occurred frequently. Perkins and associates[135], in 1950, described a case of vacuolar nephropathy associated with chronic diarrhea and they were the first to point out that hypokalemia is etiologically related to the renal lesion.

The vacuoles develop chiefly within cells of the proximal convoluted tubules (Figure 1), but can also involve more distal parts of the nephron. Occasionally, they are found in the parietal epithelial cells of Bowman's capsule[95, 167]. They vary in size and are often located below the nucleus. The hyaline droplet degeneration noted in the kidneys of experimental animals (*vide infra*) may also occur in man[145]. However, unlike in experimental animals, hyperplasia of epithelial cells in corticomedullary tubules is not a feature of human kaliopenic nephropathy. The renal lesion has been noted in as many as sixty per cent of cases of potassium depletion[81]. However, in another series of fifty cases of prolonged hypokalemia[124], the incidence was only fourteen per cent. The apparent discrepancy is not surprising, inasmuch as the tubular vacuolation has been shown to be a reversible process[140], and in many of the reported cases in which the lesion was not found only renal biopsies were examined.

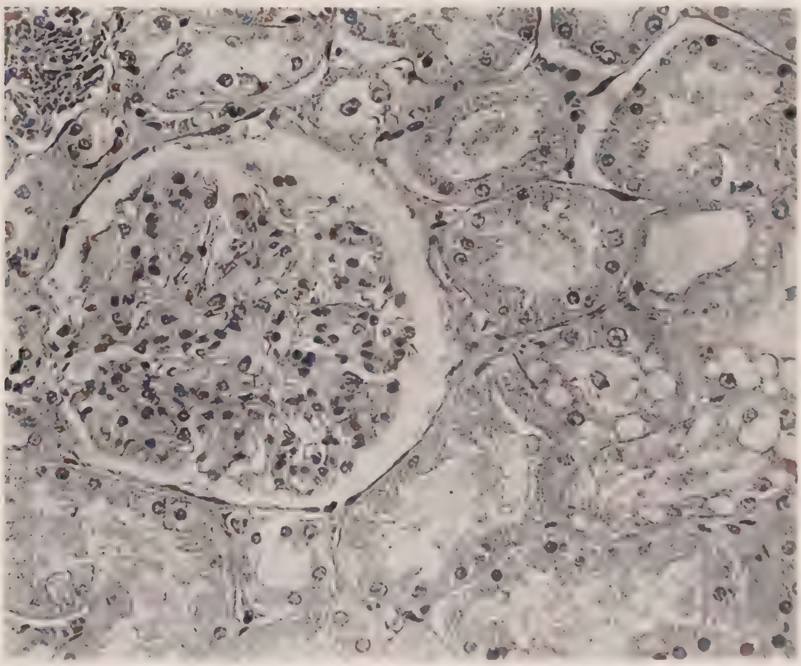

Fig. 1. Focal vacuolar nephropathy in a man with hypokalemia secondary to gastrointestinal disease (200 x).

Muehrcke and McMillan[124] reviewed thirty-five reported cases of prolonged hypokalemia in man and described fifteen of their own. The causes of the potassium depletion in these patients are listed in Table I. The concentrations of

TABLE I CAUSES OF POTASSIUM DEFICIENCY IN FIFTY REPORTED CASES OF PROLONGED HYPOKALEMIA[124]

GASTROINTESTINAL:	19 cases	
Diarrheas	12	(nonspecific, self-induced, due to excessive use of laxatives or enemas)
Steatorrhea	3	
Ulcerative Colitis	1	
Zollinger-Ellison	1	
Thyrotoxicosis	1	
Vomiting	1	
RENAL: 30 cases		
Primary renal tubular losses	17	(including cases of renal tubular acidosis and one each of Fanconi syndrome and cystinosis)
Aldosteronism	11	
Adrenocortical Hyperplasia	1	
Malignant Hypertension	1	
IDIOPATHIC: 1 case		

potassium in the serum ranged from 1.1 to 2.8 mEq. per L. In the fifteen cases studied by the authors, the duration of hypokalemia had varied from more than three months to thirteen years. The most common urinary abnormality was proteinuria, noted in forty-one of forty-four cases. Bacteriuria was detected in nineteen of thirty-seven. Cultures of the urine yielded growth of enterococci in one case, Staphylococcus albus in another, and gram negative bacilli in the rest. The histopathologic changes in the kidneys are listed in Table II. Notwithstanding the difficulties implicit in the histologic diagnosis of pyelonephritis, it is evident that this disease occurs frequently in patients with prolonged deficiency of potassium. The relation of potassium depletion to bacterial infection of the kidney is discussed below. Vacuolar nephropathy also has occurred in association with bacillary dysentery[34, 88], regional enteritis[95], diverticulitis[95], Whipple's disease with concomitant parathyroid hyperplasia[128], cholera[16], cholangitis[95], hydrocephalus[4], and following the administration of 9-α-fluorohydrocortisone[183] or chlorthiazide.[125]

TABLE II RENAL PATHOLOGIC CHANGES IN THE FIFTY CASES LISTED IN TABLE I

No information	5
No histologic studies	6*
Normal biopsy	1
Chronic pyelonephritis	18
Chronic and acute pyelonephritis	2
Acute pyelonephritis	1
Interstitial fibrosis	5
Vacuolation tubules	4
Interstitial fibrosis and vacuolation	3
Benign nephrosclerosis	2
Malignant nephrosclerosis	1
Hydronephrosis	2

*In four of these patients roentgenograms of the abdomen revealed nephrocalcinosis.

Two electron microscope studies of kaliopenic nephropathy in man have been published. Biava and others[17] examined sections from a case of aldosteronism. They found vacuoles in proximal and distal convolutions as well as in the terminal ascending segments of Henle's loops. The vacuoles were located within invaginations of the basilar cell membranes, normal components of the cell which rest on the basement membrane and communicate with the extracellular space. The authors interpret the vacuolation as an expansion of the extracellular space. Similar expansions of basilar lamellae in the tubular cell membranes have been noted in the rat after neurohypophysectomy[96], under conditions of active transport of substances like diodrast from peritubular capillaries to tubular lumens[144], following the intravenous injection of isotonic saline solution[31], and in uranium poisoning[163]. They also have occurred in rabbits depleted of sodium and chloride and given glucose intravenously[146]. Muehrcke and Rosen[125] studied biopsies from six patients who had had hypokalemia for periods varying from two months to twenty-seven years. In five cases, the vacuoles were small and seemed to originate from the infolded basilar membranes. The vacuoles noted in the sixth case, which had the severest degree of hypokalemia, were large but unassociated with separation of the basilar membrane. The authors concluded that such vacuoles are not necessarily expansions of the extracellular space.

Depletion of potassium in experimental animals has been produced most frequently by means of deficient diets. It also has been achieved by the administration of cation exchange resins, desoxycorticosterone acetate or sodium bicarbonate. The last method leads to potassium deficiency through an increase in the urinary excretion of the cation. The depleted animals do not gain weight as well as pair-fed controls, and they frequently develop diarrhea, paralyses and myocardial lesions. Pathologic changes in the kidneys were first described in mice[99] but occur most consistently in rats. They also have occurred in rabbits[91], monkeys[153a], and in about twenty-five per cent of potassium-depleted dogs studied[81]. In the experiments of Kennedy and Parker[90], old rats developed renal lesions more readily than young rats, but the degree of potassium deficiency was not specified.

In some of the initial studies on experimental potassium depletion[62, 167], the acute renal lesions were found to consist of tubular ectasia and cytoplasmic degeneration; tubular necrosis and calcification developed if the depletion was prolonged. These findings have not been corroborated by subsequent investigations. The reasons for this are unknown, although several explanations can be offered. Tauxe and others[167] used weanling animals, but no differences in susceptibility based on age alone have been delineated. In the majority of recent experiments, the earliest renal lesion has been a type of hyaline droplet degeneration confined to the inner medulla and papillae. This lesion was first noted in mice, but it is seen best in rats. The hyaline granules measure 0.2 to 4 microns in diameter and are located within epithelial cells of the collecting tubules as well as in the adjacent interstitial cells and capillary endothelium. They are stained by the periodic acid-Schiff stain, and this is not abolished by prior treatment of the tissue with amylase. The Hale iron reaction is also positive[65], which suggests the presence of acid mucopolysaccharides. The latter reaction is unaffected by hyaluronidase but is abolished by neuraminidase, an enzyme which attacks the sialomucins of *abnormal* rat tissues[65]. Five studies of the hyaline granules with the electron microscope have been reported. In a preliminary note, Muehrcke and Bonting[123] described the occurrence of

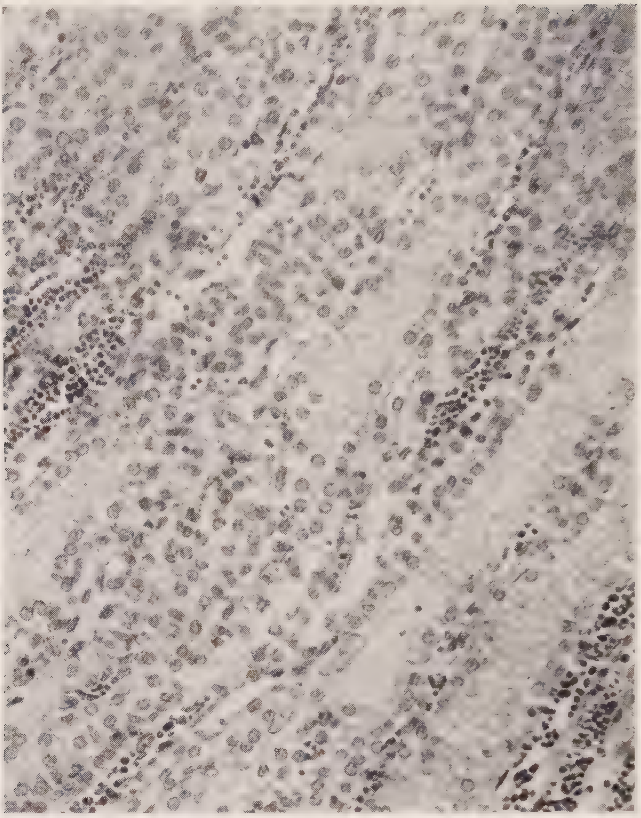

Fig. 2a

marked changes in the mitochondria of collecting tubules and distal papillae, consisting of swelling followed by fragmentation and release of osmiophilic material into the cytoplasm. In a more recent report, Muehrcke and Rosen[125] state that although some of the osmiophilic bodies contain mitochondria, "one cannot conclude that (they) originate from this source." The bodies seem to be enclosed by a single membrane, and they vary in size, structure, and degree of osmiophilia. MacDonald and associates[104] thought that the osmiophilic granules had developed chiefly within mitochondria. On the other hand, the studies of Spargo *et al.*[160] and of Morrison and Panner[120, 121] seem to indicate that the particles in question originate from lysosomes. Additional evidence of an origin from lysosomes is found in the studies of Morrison and Panner, who measured the activity of acid phosphatase in the renal papillae of potassium-depleted rats and found levels much higher than in the control animals. Wachstein and Meisel[177], using histochemical technics, also detected increases in the activity of acid phosphatase in the collecting tubules and papillae.

The hyaline granules which develop in the renal tubules of rats after the administration of foreign protein also have been variously interpreted as originating from mitochondria[132] or lysosomes[163a]. Oliver and MacDowell[130] have shown that the hyaline droplets found in the convoluted tubules of rats after the intraperitoneal injection of foreign protein are stained by T-1824 if the dye is given simultaneously. Morrison and Gardner[118, 119] used this method to study the extent to which tubular reabsorption of serum protein contributes to the formation of the hyaline granules in potassium depletion. In control rats, the dye was seen chiefly in droplets within cells of the cortical convoluted tubules, whereas in depleted rats it appeared in greater amounts within the cells of collecting tubules. Since the deficient rats also developed significant proteinuria, the authors concluded that the depletion of potassium probably had diminished the capacity of the renal tubules to absorb protein.

As the severity of hypokalemia increases, a second lesion develops in the kidneys of depleted animals: hyperplasia of epithelial and intercalated[131] cells in convoluted tubules of the outer medulla and inner cortex (Figure 2). Mitoses are seen frequently. Swelling of the lining cells may block the lumen of the tubule, thus producing ectasia of proximal parts of the nephron. Using microdissection, Holliday *et al.*[84] found epithelial hyperplasia in some of the proximal tubules. The distal tubules are not affected.

TABLE III HISTOCHEMICAL AND BIOCHEMICAL ASSAYS OF ENZYME ACTIVITY IN EXPERIMENTAL POTASSIUM DEPLETION

I. HISTOCHEMICAL STUDIES
- (1) Alkaline Phosphatase
    - Normal[36, 133]
    - Decreased[122, 159]
    - Focal decreases[177]
- (2) Acid Phosphatase
    - Normal[159]
    - Increased in collecting tubules[36, 133, 177]
- (3) Nonspecific Esterase (collecting ducts)
    - Normal[36, 159]
    - Increased[133]
- (4) Succinic Dehydrogenase
    - Normal[36, 133]
    - Decreased[177]
- (5) 5-Nucleotidases
    - Normal[36, 133]
- (6) Lactic Dehydrogenase
    - Increased collecting ducts and decreased in loops of Henle[123]
- (7) Diaphorases
    - *TPN* diaphorase high and *DPN* diaphorase low in medulla and papillae[133]
    - Increase both diaphorases[177]
- (8) Carbonic Anhydrase
    - Increased in Rats[56]
    - In dogs—decreased when induced with *DOCA* but increased when induced with HCO_3[87]

II. Biochemical Studies
- (1) Alkaline Phosphatase[123]
    - Low in blood vessels, glomeruli, inner medulla and base of papillae
    - Normal in proximal tubules, outer medulla and tips of papillae
- (2) Lactic Dehydrogenase[123]
    - Low in glomeruli, cortical proximal tubules and papillae
    - Normal in proximal tubules of outer medulla and in blood vessels
    - Increased in tubules inner medulla
- (3) Glutaminase
    - In rats[86], increase in units per gram of kidney nitrogen
    - In dogs[87], increased only when supplemental DOCA was given
- (4) Pyruvate Kinase[40]
    - Increased amount, confined to medulla, compensated for the lowered enzyme activity which follows K+ depletion

Histochemical and biochemical analyses of the kidneys of potassium-deficient animals have yielded conflicting results, some of which are summarized in Table III. Variations in the findings may be partly due to differences in susceptibility among animal species, to undetected differences in experimental technique, and to the difficulties inherent in histochemical analyses in general. Animals depleted of potassium develop not only

Fig. 2. (a and b): Foci of hyperplasia of epithelial cells in tubules of the outer medulla of rats depleted of potassium for a period of two weeks (200 x).

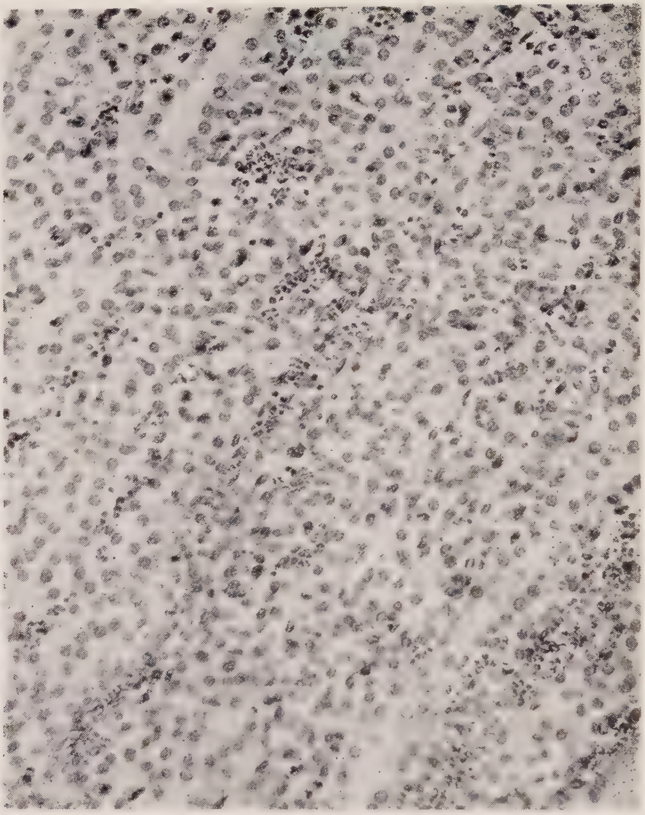

Fig. 2b

hypokalemia but also a marked decrease in the concentration of potassium in muscle. This occurs even when the potassium levels in the kidneys are normal[39, 87]. Reduction in the concentration of the cation in the kidneys is not nearly as marked as the concomitant reduction in muscle[126]. Wilson and Kissane[186] found decreased potassium levels in the papillae and inner medulla but normal levels in the cortex. In another study[108], the levels of potassium were decreased in the papillae and the cortex but were normal in the medulla. Unlike in muscle, the decrease in the potassium concentration of the kidneys is not accompanied by an increase in the concentration of sodium[25, 108].

The kidneys of animals depleted of potassium are larger than normal[40, 45]. Davis[40] found the ratio of *DNA* phosphorus to fat-free dry solids to be the same as in controls, and he interpreted this as evidence that hyperplasia is the main cause of the renal enlargement. The total amount of water and solids is increased, either in equal degree[86, 159] or with a greater increases in water[25, 134]. An increase in the amount of basic amino acids has been detected in rats[23, 46, 47, 85a], but not in dogs[85]. The amount of fat per total renal mass seems to be the same as in control animals[25, 139]. Anderson and Mudge[1] noted the occurrence of intracellular acidosis. This is compatible with the demonstrated impairment in the renal excretion of bicarbonate. Cooke[35a], who mentions the occurrence of aminoaciduria in potassium-deficient animals, has noted marked increases in the amount of diamino acids in renal homogenates.

The renal lesions associated with acute potassium depletion disappear when the depleted stores are replenished[82, 99, 115, 160, 167]. The hyaline-droplet lesion tends to disappear first, usually within one to two weeks, but occasionally as promptly as two to three days. Disappearance of the hyperplastic lesion may sometimes leave residual cortico-medullary scars[82]. Pearse and McPherson[133] found that the activities of most enzymes had returned to normal one week after repletion. Rubidium, and to a lesser extent cesium, can apparently prevent the renal damage, or can accelerate its repair[60].

Prolonged cyclic depletion of potassium is much more likely to produce residual interstitial fibrosis of the renal medulla and papillae than acute depletion. Fourman, McCance and Parker[63] found severe residual lesions as long as seven months after repletion; the kidneys were markedly enlarged, and tubular ectasia was prominent. A similar ectasia of corticomedullary tubules has been noted in rats fed high-protein diets, and in old rats conditioned early in life to a habitually high oral intake of water[59]. Perdue and Phillips[134] found that the incidence of renal tubular ectasia and myocardial necrosis in potassium-deficient rats was higher when the diet contained twenty per cent corn oil than when its content of corn oil was five per cent.

There is good evidence that the residual lesions of potassium deficiency in the kidneys of man[124] and experimental animals[187] can render the kidneys abnormally susceptible to bacterial infection. In this respect, it is thought that the scars in the medulla may be a *locus minoris resistentiae*. Beeson and associates[15] produced tiny intrarenal scars in rats by means of cautery. Cortical scars, even when extensive, did not make the kidneys more susceptible to infection; however, as few as a single medullary scar increased the incidence of pyelonephritis after the intravenous injection of bacteria. A similar effect of thermal trauma has been observed by Rocha and Fekety[142]. In this respect, the effects of trauma and hypokalemia may be additive[24]. According to Muehrcke[122], lack of potassium also favors the dissemination of infection through and beyond the kidney. The increase in susceptibility to infection also may be related to the increase in the renal production of ammonium ions which occurs as a result of potassium depletion. The renal tubular ectasia characteristic of prolonged potassium depletion reflects a state of intrarenal hydronephrosis[41], which would also predispose to infection. The ectasia is probably due to multiple factors such as distal obstruction of tubular lumens by hyperplastic epithelial cells or casts, the attendant polyuria, and a decrease in ureteral peristalsis. The latter has been found to occur in dogs deficient in potassium[150]. Although potassium depletion can occur as a result of acute or chronic renal disease[112], it is not a common sequel of uncomplicated chronic pyelonephritis. Many cases of *potassium-losing nephritis* are actually instances of aldosteronism. Carroll and Farber[30] have reported on the occurrence of spontaneous *hyperkalemia* and hyperchloremic acidosis in patients with chronic pyelonephritis.

There seem to be few other renal sequelae of prolonged potassium deficiency, although Grollman and White[70] found significant systolic hypertension in one-year-old rats which, as weanlings, had been given diets deficient in potassium for twenty-one days.

The most frequent abnormality of renal function associated with hypokalemia is impairment in the ability of the kidneys to concentrate the urine. This defect, which is not corrected by antidiuretic hormone, occurs in man[50, 140, 147], dogs[158], rats[67, 115, 141], and probably also in rabbits[81]. In one report[92], the impaired urinary concentration was thought to have been due both to the renal lesions and to a transient state of diabetes insipidus. In man[147] and in rats[98], it develops without concomitant impairment in the renal dilution of urine. When rats are depleted of potassium acutely (within a period of four weeks or less), the degree of impairment in the renal concentration of urine parallels the severity of the potassium depletion but not its duration. On the other hand, when the depletion of potassium is prolonged more than sixty days, the severity of the defect can be correlated better with the duration of the depletion[181]. This defect is not merely a reflection of the coexisting polydypsia[21], nor is it dependent on abnormalities of other serum electrolytes. It disappears when the stores of potassium are replenished: in eleven cases mentioned by Hollander[81], normal renal function was restored from one and one-half months to four years after repletion. The correction of this defect generally coincides with the disappearance of the hyaline droplet lesion in the tubules of the inner medulla.

Animals depleted of potassium tend to develop metabolic alkalosis and excrete urine which is inappropriately acid. The renal excretion of bicarbonate is impaired: Anderson and Mudge[1] found that slices of renal cortex from potassium-depleted rabbits take up less bicarbonate from solutions of sodium bicarbonate than control slices of normal rabbit kidney. Holliday and others produced potassium deficiency in rats by inducing alkalosis and found severe degenerative changes in the proximal tubules. However, in a subsequent experiment, they found similar lesions of the proximal tubules in animals depleted of chloride alone and in the absence of alkalosis[83]. Failure to produce renal lesions consistently through the induction of alkalosis has also been reported by other investigators[127, 185]. The alkalosis which develops in dogs depleted of potassium *and* chloride is corrected completely only upon repletion of both electrolytes[6]. Potassium depletion increases the severity of the lesions caused by magnesium depletion[156] and by phosphate loading[84a].

The following additional abnormalities of renal function have been found to occur in man as a result of potassium deficiency:

(a) Renal phosphaturia with hypophosphatemia[106].

(b) Uremia[171].

(c) Decreased glomerular filtration rate and decreased urea clearance[140].

(d) Impaired extraction and excretion of p-aminohippurate[140, 165].

(e) Decreased excretion of citric acid and other organic acids[51].

(f) Impairment in the capacity to

conserve sodium with diets low in sodium[106].

SODIUM AND CHLORIDE

Hypernatremia and Hyperchloridemia

Few histopathologic studies have been published concerning the effects of hypernatremia and hyperchloridemia on the human kidney. When they are the result of adrenocortical hyperfunction, the attendant hypokalemia and the hormonal imbalance preclude adequate study of the specific effects of excess sodium and chloride. The majority of cases suitable for study are infants with hypernatremia secondary to gastrointestinal disease, and cases in which large amounts of sodium chloride were ingested[48, 54, 151, 153, 180]. Evaluation of the renal changes in these instances should include a consideration of the possible independent effects of dehydration.

Arey and Reardon[5] compared the histologic changes in the kidneys of eight infants with hypernatremia secondary to nonrenal lesions with the renal histologic changes in 297 infants who had died without evidence of electrolyte imbalance. Dilatation of the cortical tubules was seen in four of the eight cases of hypernatremia but occurred in only 9 per cent of the control group. The presence of hyaline casts in the lumens of the ectatic tubules was noted much more frequently in the group with electrolyte imbalance. The findings are of interest, but the number of cases is small. According to Finberg[53], dilatation of cortical tubules with luminal casts occurs in kittens with hypernatremia but is seen also in association with dehydration alone. Rush and associates[148] studied dogs subjected to extracorporeal dialysis and compared the effects of hypernatremia with the effects produced by hypertonic solutions containing normal concentrations of electrolytes. In the dogs made hypernatremic, the fluid bathing the artificial kidney contained 205 mEq. per L. of sodium, 165 mEq. per L. of chloride, and its total osmolality was 430. In the other group of dogs, the concentrations of electrolytes in the bathing fluid were normal, but the total osmolality was increased to 415 by the addition of urea. Renal lesions were noted only in association with hypernatremia, and consisted of cystoplasmic vacuolation or necrosis of the convoluted tubules, chiefly in the proximal segment.

Lesions in the central nervous system are usually the severest and most constant changes in cases of intoxication following the ingestion of large amounts of sodium chloride. This has been noted in human beings[102, 103] as well as in dogs[148], cats[55], poultry[157], and swine[157]. The brains are shrunken and have multiple areas of thrombosis and hemorrhage. In the experiments by Rush and associates cited above, the dogs dialyzed with a hypertonic solution containing urea but normal concentrations of electrolytes did not suffer cerebral hemorrhage or thrombosis, although their brains were moderately shrunken. Venous thrombosis also has been noted in the kidneys[5, 48]. Acute necrotizing arteritis has been reported in rats[170] and occurs occasionally in man[48]. In swine, the lesions also have included gastritis, cystitis, splenic infarcts, necrosis of the glottis and degenerative changes in the liver[157]. Elton and associates[48] have described the renal lesions in six infants who died as a result of accidental intoxication with sodium chloride. The proximal and distal convoluted tubules were shrunken due to a marked extracellular accumulation of fluid between the cell membranes and the basement membranes. No definitely intracellular vacuolation was seen. In three cases, the collecting tubules contained amorphous pink casts. Similar casts were noted in the cases described by Luttrell and Finberg[103] and are mentioned in the report by Arey and Reardon[5]. Baxter and Teschan[14] have described a case of hypernatremia, secondary to heat stroke, in which there developed acute renal failure and fulminant intoxication with potassium.

The administration of excessive amounts of sodium chloride, singly or in conjunction with desoxycorticosterone acetate, has been used many times in the experimental induction of hypertension. Genetic factors may play a role in determining the susceptibility of some experimental animals to the development of hypertension, secondary to excessive ingestion of salt[38]. Hall and Hall[71] recently reported on the effect of increased ingestion of salt on adrenal-regeneration hypertension.

Hypochloridemia and Hyponatremia The administration of diets low in chloride to rats was found to cause precipitation of calcium salts in the collecting tubules, an effect which was attributed to the coexisting alkalosis[100]. No constant lesions occurred in the other organs. Other studies have reported impairment in the growth of rats as a result of chloride deficiency. The subject has been reviewed by Follis[61].

The main histologic change in the kidneys as a result of sodium deficiency seems to be an increase in the granularity of the juxtaglomerular cells located in the media of the afferent arteriole. During the last decade, there has been a tremendous resurgence of interest in the juxtaglomerular apparatus of man and experimental animals. Since it is not possible to discuss adequately the vast amount of work published, the reader is referred to several general reviews[114, 138, 164, 168] of the subject, as well as to reported studies on morphology[9, 10, 11, 44, 64, 97], computation of juxtaglomerular indices[73] and cell counts[173], histochemical analyses of enzyme activity in the macula densa[56, 77], effects of sodium depletion[28, 57, 110, 137] or retention[73, 137, 169, 174], and assays of renal tissue for renin or renin-like activity[18, 32, 117]. A promising technique in the study of normal and abnormal renal function is the intracellular localization of sodium[113] (and potassium[89]) by means of autoradiography.

Administration of diets low in sodium causes an increase in the granularity of the juxtaglomerular apparatus. This has been noted in man as well as in the dog, cat, rat, mouse and rabbit. This increase is not prevented by the administration of desoxycorticosterone acetate. The increase in the juxtaglomerular index of granularity is directly proportional to the duration of the sodium deficiency and occurs before the serum concentration of sodium falls. A high dietary intake of sodium tends to lower the juxtaglomerular index, which is decreased further by the concomitant administration of desoxycorticosterone acetate. An inverse correlation between the serum concentration of sodium and the extent of granularity of these cells can be demonstrated best in the rat and in the cat. There is considerable variation in the normal number and prominence of juxtaglomerular granules in different animal species. In our experience[4], it has been difficult to obtain reproducible results with human kidneys. Several investigators[56, 77] have studied histochemically the activities of certain enzymes in the macula densa involved in the hexose monophosphate shunt, specifically glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. In general, the activities of these enzymes parallel the degree of granularity of the juxtaglomerular cells, but the relation is not quantitative. Increases in the activity of glucose-6-phosphate dehydrogenase in proximal convolutions have been noted in association with potassium deficiency.

CALCIUM

Nephrocalcinosis was discussed at some length in the Applied Seminar on Thyroid and Parathyroid Functions[2] and will not be reviewed here. Epstein[49] has reviewed the effects of hypercalcemia on renal function. Table IV lists some clinical disorders associated at times with calcification of the renal parenchyma. Although it is primarily a radiologic diagnosis, nephrocalcinosis may not be detected in the roentgenograms and still cause (or contribute to) impairment of renal function. The calcium deposits in the kidney may regress or disappear completely if the underlying disorder is corrected. Prolonged hypocalcemia apparently does not produce distinctive lesions in the human kidney, since no renal abnormalities have been noted in cases of hypoparathyroidism or pseudohypoparathyroidism. No studies on the specific renal effects of hypocalcemia in experimental animals could be found in a perusal of the medical literature.

TABLE IV SOME DISORDERS ASSOCIATED WITH MICROSCOPIC CALCIFICATION OF THE RENAL PARENCHYMA[3]

ENDOCRINE
- Hyperparathyroidism
- Hyperthyroidism

METABOLIC
- Renal tubular acidosis (especially in the adult)
- Fanconi syndrome
- Wilson's disease
- Syndrome of renal tubular disease, muscle paralysis and hypokalemia (Owen and Verner)
- Oxalosis
- Hypophosphatasia
- Idiopathic hypercalciuria
- Osteoporosis
- Milk-alkali syndrome
- High intestinal obstruction, hyperventilation syndrome and other disorders accompanied by alkalosis
- *Blue diaper* syndrome[42]

INTOXICATIONS
- Vitamin D
- Bichloride of mercury, sulfonamides and other nephrotoxins

NEOPLASMS
- Multiple myeloma
- Other malignant tumors (with or without demonstrable bone metastases)

MISCELLANEOUS
- Sarcoidosis
- Chronic pyelonephritis
- Chronic glomerulonephritis
- Idiopathic hypercalcemia of infancy (many of these cases are probably caused by intoxication with vitamin D)
- Congenital renal tubular ectasia *(sponge kidney)*
- Fibrous dysplasia of bone
- Osteitis deformans
- Following recovery from bilateral renal cortical necrosis

IDIOPATHIC

PHOSPHATE

Phosphate-loading in the rat causes necrosis of the proximal convoluted tubules. By microdissection of nephrons, Oliver[129] was able to localize this lesion to the terminal portions of the proximal tubules and to isolated segments of the ascending limbs of Henle's loops. These changes occur independently of the cation given with the phosphate and the pH of the salt. It has been reported that phosphate-loading aggravates the renal lesions caused by potassium depletion[156]. However, in carefully controlled studies, Holliday *et al.*[84a] have shown that the renal lesions in animals deficient in potassium and given excessive amounts of phosphate resemble those seen in phosphate-loading alone. It would seem, therefore, that it is potassium deficiency that aggravates the renal lesions caused by phosphate-loading and not vice versa. Although it has been claimed that the excessive administration of phosphate will lead to parathyroid hyperplasia, this does not occur if the dietary intakes of calcium[162] and vitamin D[61] are adequate. The nephrocalcinosis and the bone changes produced in rats injected with parathyroid extract have been potentiated by the concomitant administration of monobasic sodium phosphate[154]. Deficiency of phosphorus in experimental animals induces excessive losses of calcium but does not interfere appreciably with the metabolism of sodium, potassium and magnesium. Diets deficient in phosphorus have produced hypercalcemia in puppies and citrate calculi in the kidneys of rats[61].

MAGNESIUM

Wacker and Vallee[179] have reviewed the subject of magnesium metabolism in man. The highest concentrations in tissue are found in striated muscle and liver. The amount normally excreted in the urine is about one third of the intake. It is filtered by the glomeruli and reabsorbed by the tubules. There is evidence that, like potassium, it may be excreted by the tubules. The occurrence of hypermagnesemia in chronic renal failure was reported first by Salvesen and others[149] in 1923, and has been noted many times since by other investigators. The administration of magnesium salts to patients with chronic renal disease may produce a marked elevation in the serum concentration of magnesium. Low levels of serum magnesium have been noted sometimes in the presence of chronic renal disease. Hirschfelder[79] described four such cases in association with chronic glomerulonephritis; in two patients, the serum concentration of calcium was notably increased. The serum levels of magnesium are frequently higher than normal in the presence of acute renal failure[72, 178]. Hamburger[72] detected hypermagnesemia in each of 220 patients with acute renal insufficiency. The average serum concentration of magnesium was 2.6 mEq. per L., and in all patients but one, the serum calcium levels were decreased.

Martin and others[109] have discussed causes of disturbances in magnesium metabolism in man. Fitzgerald and Fourman[58] tried to produce magnesium deficiency in two volunteers who consumed deficient diets for twenty and twenty-seven days. The levels of serum magnesium did not fall, but there occurred retention of sodium and chloride. Hypocalcemia and hypophosphatemia occurred frequently in ten patients with magnesium deficiency reported by Randall and associates[139]. The patient studied by Petersen[136] had hypocalcemia and increased intestinal losses of sodium and potassium. The addition of magnesium supplements to the standard diets of normal persons leads to an increase in the urinary excretion of calcium[33, 74]. There are practically no data on the effects of disturbances in magnesium metabolism on the structure of the human kidney. In the ten cases of magnesium deficiency reviewed by Randall and associates, no renal lesions were found. Sporadic reports of magnesium intoxication in man have been published[35, 52, 161], but the histopathologic changes in the kidneys have been absent or mild and nonspecific.

The signs of magnesium deficiency in experimental animals were originally described by Kruse, Orent and McCollum[94]. The early changes consist of peripheral vasodilatation and hyperemia, particularly noticeable in the nose, ears and foot pads. Generalized perivasculitis occurs frequently in the early stages of deficiency. Bloody diarrhea is not unusual and develops, as a rule, after the first week of depletion. Increased neuromuscular irritability and convulsions may be noted when the duration of depletion has exceeded four weeks. At necropsy, lesions of the heart and central nervous system[19] are frequent; skeletal muscle may be degenerated or inflamed; the bones may appear brittle[182]; and the gingival connective tissue may be hypertrophied[61]. Moore, Hallman and Sholl[116] found calcification of the endocardium and the elastic tissue of blood vessels in calves. Heggtveit and associates[75] describe the occurence of myocardial necrosis and calcification in rats, a lesion found sometimes in association with potassium deficiency[155]. The effects of magnesium and potassium depletions may be additive[84a, 156]. Magnesium seems to be an essential dietary requirement for normal growth of guinea pigs[143]. The peripheral vasodilatation which occurs in animals deficient in magnesium may be accompanied by a rise in the levels of histamine in blood and urine. Bois[22] noted the occurrence of degranulation of mast cells in rats, but this event did not parallel the increases in the levels of histamine in the time of occurrence or in intensity.

The earlier studies of the renal histopathologic changes in rats[26, 37, 68, 69] and rabbits[12] describe a variety of degenerative changes in cortical and medullary tubules. However, Blaxter, Rook and MacDonald[20] found no distinctive lesions in the kidneys of bull calves which, for a long time, had eaten diets deficient in magnesium. More recent experiments on magnesium depletion in the rat[13, 93, 101, 184] indicate that the basic renal lesion is the formation of calcium casts without primary changes in the epithelial cells of the tubules. This has been confirmed with the electron microscope[13]. Welt[184] has found that the luminal deposition of calcium is confined to the broad ascending limbs of Henle's loops in the outer medulla. This specific localization of the calcium casts contrasts with the diffuse nephrocalcinosis which follows phosphate-loading or intoxication with vitamin D. Residual casts were noted as long as six months after depleted stores of magnesium had been replenished[184]. Calcification is also a frequent occurrence in the myocardium[75]. Hellerstein and associates[76] induced magnesium deficiency in rats fed an atherogenic diet for about twenty-five days. It was necessary to feed the animals eight to fifteen times the normal dietary requirement of magnesium in order to prevent completely the development of renal calcinosis. The addition of thyroxine to the diet did not change the low serum levels of magnesium but decreased greatly, or eliminated, the renal lesion. Reference has been made above to the effects of a high fat diet on the experimental lesions associated with potassium depletion.

Injury to mitochondria seems to be an important effect of magnesium deficiency, although electron-microscope studies of rat kidneys by Battifora and associates[13] revealed no abnormalities in these structures as long as four weeks after the onset of magnesium deprivation. Magnesium protects isolated mitochondria of rat liver from the effects of agents which tend to cause swelling and to uncouple oxidative phosphorylation, such as thyroxine[166]. Gershoff and associates[66] have studied some interrelations in the metabolism of magnesium, thyroxine and vitamin B_{12}. Isolated mitochondria of rat liver swell when placed in a medium free of magnesium[8]. Vitale and others[176] detected uncoupling of oxidative phosphorylation in mitochondria from the heart, liver and kidneys of magnesium-deficient rats.

The histochemical studies carried out by Hess *et al.*[78] revealed an increase in the activities of succinic dehydrogenase and in the *DPN* and *TPN* diaphorases in the terminal segments of the proximal convoluted tubules early in the course of magnesium deprivation. On the other hand, Ko and associates[93] noted a progressive fall in the activities of these enzymes, in addition to decreases in alkaline phosphatase, acid phosphatase and nonspecific esterase. Watchorn and McCance[182] found a normal activity of the phosphatases in blood, bone and kidney.

Although hypomagnesemia is the rule in animals depleted of magnesium, the serum concentrations of calcium have been variously reported as low[27, 29, 111], normal[20, 43, 93, 94, 172, 175] or high[78, 105]. The concentration of magnesium within erythrocytes was found to fall to about half the normal values[172]. The activity of serum alkaline phosphatase has been reported as normal[182]. The serum concentrations of sodium and potassium are usually normal, although the occurrence of sodium retention has been described[105]. The concentration of potassium in muscle is often decreased[105, 107, 184]. Urinary excretion of potassium was found to be increased by Welt[184], but was normal in the studies of Manitius and Epstein[107]. Unlike deficiency of potassium, magnesium deficiency does not cause increase in the urinary excretion of ammonia and does not impair the capacity of the kidney to concentrate the urine. The kidneys of experimental animals deprived of magnesium contain greater than normal concentrations of calcium[78, 105, 172, 182, 184] and normal[78, 105] or decreased[182] concentrations of magnesium. Proteinuria[69], hypoproteinemia[68, 93] and the nephrotic syndrome[12, 68] have been noted in some studies. The levels of calcium in heart[172] skeletal muscle[172] and soft tissue[27, 29, 111] are often increased but may be normal[105]. The most frequent change in the magnesium concentration of other tissues has been a fall in the levels in bone[20, 105, 182]. The magnesium levels in muscle, brain and soft tissue have been usually normal[20, 105, 182], although decreases have been noted[105]. The studies of Welt[184] indicate that in rats with magnesium deficiency there is normal urinary excretion of calcium and an increase in its absorption by the intestine. This suggests that in magnesium depletion there occurs either a redistribution of calcium between the extracellular fluid and some other tissue compartment or some change in the affinity for calcium of protein-binding sites. Increased activity of the parathyroid glands may play a role in producing this change. Parathyroid hyperactivity in magnesium depletion is also suggested by the reported occurrence of increased urinary excretion of phosphate[184].

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Disturbances of Fluid and Electrolyte Metabolism in Intestinal Obstruction

WALTER F. BALLINGER, II, M.D.

Alterations in measured volumes of fluid and the concentration of electrolytes in the various body compartments have been the subject of intensive investigation for many years. Initially, such studies were confined to changes which were directly detectable in whole blood or serum. From these data, assumptions were made as to shifts from intracellular and interstitial compartments in and out of the vascular space. Studies were also initiated to determine not only the quantity but also the composition of the fluid contained within the lumen of the obstructed intestine. It has become appreciated that changes in fluid and electrolyte concentration will vary according to the cause, type, and location of the obstruction and according to the rapidity with which secondary changes progress. In the past few years, investigators have concentrated upon transport mechanisms involved in the flux of water and electrolyte across the intestinal mucosa, not only from lumen to vascular compartment, but also in a reversed direction from the vascular space to the lumen. Thus, contemporary understanding requires not only an evaluation of the changes as determined in whole blood and serum, but also an understanding of the bi-directional flux of water and electrolytes and the transport mechanisms involved, as part of the pathophysiology of intestinal obstruction.

The relative frequencies of the causes of intestinal obstruction have altered in this century, as the peritoneum has been transgressed more often by the surgeon. Today, adhesions account for almost one half of all cases of obstruction of the small intestine. Neoplastic and inflammatory obstructions are more common in the colon. The causes of obstruction of the small intestine and

TABLE I CAUSES OF OBSTRUCTION OF THE SMALL INTESTINE

	Per cent
Adhesions	43
External hernia	32
Tumor	6
Congenital anomalies	6
Inflammatory disease	6
Volvulus	3
Intussusception	2
Miscellaneous	2

their relative frequency are listed in Table I. These figures are strikingly similar from clinics all over the country. Although these figures are of interest, especially to the clinician, it is more important and more convenient to divide the obstructions into adynamic or mechanical, simple or strangulated. In Table II, the more common types are listed. Furthermore, it is of significance that forty per cent of intestinal obstructions are of the simple variety, whereas sixty per cent are of the strangulated type. Thus, actual vascular embarassment exists in over half the cases of intestinal obstruction from the very beginning. It should be recognized, however, that as distention progresses, even in the cases of simple intestinal obstruction, increased intraluminal pressure will result in venous occlusion within the wall of the distended gut and that this vascular change will progress proximally in the presence of an unrelieved block. Basically then, obstruction, from whatever the cause, results in an accumulation of gas and fluid within the lumen of the small intestine. Distension results and considerable investigation has been carried out in recent years in order to determine the effect of this distention upon secretion and absorption of the mucosa of the intestine. These changes eventually are reflected in the vascular, interstitial and intracellular compartments and produce the classic alterations in electrolytes with which we have become familiar in intestinal obstruction. Two distinct patterns are apparent. With high obstruction, perhaps due to cicatricial stenosis at the pylorus, losses of large quantities of fluid rich in H^+ and Cl^- and lesser amount of K^+, a hypochloremic, hypokalemic alkalosis develops. Dehydration causes increases in the concentrations of these ions, but such changes become eirduct from the clinical dehydration and concomitant elevations of hematocrit and concentrations of hemoglobin. When the obstruction is low in the terminal ileum, loss of pancreatic and biliary fluid, together with succus entericus, produces a metabolic alkalosis, hyponatremia, hypochloremia and hypokalemia. Obstructions at intermediate levels may produce a balance loss with little change in the carbon dioxide combining power. However, losses of both anions and cations may be severe. Table 3 summarizes these changes.

TABLE II TYPES OF INTESTINAL OBSTRUCTION

Adynamic or Paralytic	
Metabolic	
Reflex	
Septic	
Mesenteric arterial or venous occlusive	
Mechanical	
Intraluminal	
Intramural	
Extraintestinal	
Simple	40 per cent
Strangulated	60 per cent

TABLE III FEATURES OF HIGH INTESTINAL OBSTRUCTION

1. Vomiting
2. Fluid losses obvious
3. Fluid losses may exceed 3 L. per day
4. Dehydration may mask acute electrolyte changes
5. Elevated hemoglobin, hematocrit, WBC, RBC
6. Elevated CO_2 metabolic alkalosis
7. Decreased Na, K, Cl

Approximately one third of total body water is secreted into the intestinal lumen, if one considers the entire volume flux from the mouth to the lower small intestine. Since less than 200 ml. are normally excreted via the feces, it becomes obvious that the mobilization of such a large volume of fluid from the gut to the vascular space requires more than a passive movement of water and ions across the mucosa. This uphill gradient requires a considerable expenditure of work with active transport of electrolytes and the necessity of an immediately available energy supply, chiefly in the form of high energy phosphates. Even water requires an expenditure of energy in its transport back and forth across the intestinal mucosa. The presence of glucose is essential for the absorption of water. The hypoxia that is encountered in intestinal obstruction exerts a profound effect upon the active transport of water and electrolytes.

TABLE IV FEATURES OF LOW INTESTINAL OBSTRUCTION

1. Fluid losses may be enormous but hidden.
2. Multiple Mechanisms:
   - a. Alimentary secretions
   - b. Distension
   - c. Blood loss
3. Variable changes in electrolytes.
4. Usually neither severe acidosis nor alkalosis until later when acidosis develops.

Previous concepts of the mechanisms of fluid loss in intestinal obstruction have held that the higher the lesion the greater the loss of fluid. According to this view, the alimentary secretions do not reach the absorbing surface of the intestine in a high obstruction, resulting in greater fluid losses. Lesions obstructing the small intestine near its terminus permit the alimentary secretions to reach a greater absorbing surface, thus preventing large losses of fluids. However, it became apparent that, in order to understand the pathophysiology of intestinal absorption in the presence of obstruction, it was necessary to learn how an obstruction affects alimentary secretions. It now appears that secretions are significantly increased in the presence of obstruction. This increased volume of fluid, then, reaches an area of the bowel where absorption of water and electrolytes is markedly diminished. Shields, in a series of elegant experiments, has demonstrated severe deficiencies in the bi-directional transport of water, sodium and potassium in the small intestine, proximal to the divided ileum. The fluid which accumulated above the obstruction was sampled by an indwelling catheter. It was of interest that during the first thirty-six hours there was an increased rate of secretion of fluid into the lumen of the gut, although there was little change in the rate of absorption. After thirty-six hours, however, the accumulation of fluid within the obstructed gut was markedly increased, both due to the continuing high rate of secretion into the lumen but also because of a sharp drop in the rate of absorption. Furthermore, Shields reconfirmed the fact that the fluid that was lost into the lumen of the small intestine was very similar in composition to that of serum. Approximately 75 ml. of fluid were lost into a 30 cm. length of ileum every hour.

Utilizing such figures as these, it could be postulated that, in man with a simple obstruction, one and one-half liters of fluid could accumulate in the bowel every hour. Thus, the entire serum volume could be lost into the gut in six hours. However, in such an obstruction, increased intraluminal pressure forces the fluid higher and higher in the intestine, to come in contact with mucosa that is still capable of absorption. Nevertheless, with time, derangements in the absorptive capacity of the mucosa of the intestine progresses proximally and, if allowed to continue, results in marked net losses of fluid and electrolyte from the vascular compartment and eventual deterioration of the patient. It is also understandable why rapid accumulation of fluid occurs in a closed loop obstruction, since fluid cannot be dispersed proximally to a reasonably healthy absorptive surface.

Although certain systemic factors may play a role which will modify the transport of water and electrolytes (e.g., increased activity of the adrenal cortex), it appears that the most significant causes for altered concentrations of electrolytes as reflected in the serum lies in local factors in the gut itself. For example, it has been shown that distension of the bowel with increased intraluminal pressure from whatever source produces marked decreases in the absorption of water. Indeed, an increase in intraluminal pressure to 50 cm. of water prevents transport of water across the mucosa. Such distension is an invariable accompanyment of intestinal obstruction and is produced by the accumulation of fluid and gas. Since an appreciable accumulation of gas usually precedes the accumulation of fluid, a cycle is initiated in which distension causes increased secretion of alimentary fluids and further distension of the gut.

Such distension may exert an effect by its concomitant reduction of blood flow in the intestinal mucosa. Although neural and hormonal factors have been implicated, there is no reasonable proof that they are of any importance. Sequestration of blood in obstructed bowel has been known to exist since the earliest investigations. Late in the disease, interstitial hemorrhage and frank bleeding into the lumen are well-known features. Thus, venous obstruction producing dilatation and edema of the villi could certainly account for an increased rate of secretion or simple transudation of fluid into the lumen and also explain the reduced rate of absorption.

The role of both exotoxins and endotoxins in the clinical syndrome of intestinal obstruction, especially of the strangulated type, is of interest. Both the exotoxin of Clostridia, alpha-lecithinase, and the endotoxins of coliform organisms are capable of producing severe systemic responses which are similar to those observed in unrelieved intestinal obstruction. Such effects, however, are of interest in the understanding of the clinical picture but are probably not responsible for the alterations of fluid and electrolytes.

Quantitation of bi-directional flows of water and electrolytes in health and disease has aided considerably in our understanding of the clinically observed alterations in blood constitutients. The intestinal mucosa has become rightfully recognized, along with the lungs and kidneys, as an organ of primary importance in the adaptation of man to his external environment. The need for early and adequate decompression of the obstructed lumen becomes clearer. Relief of vascular embarrassment, either arterial or venous or both, becomes paramount. Serum electrolyte changes are reflections of advanced disease involving this essential organ, the small intestine, and correction of fluid and electrolyte losses must be accomplished. The role of the absorption of toxins, especially from the strangulated obstructed bowel, still requires clarification. Advances in the understanding of the pathophysiology of intestinal obstruction have provided further evidence for the urgency in instituting early and adequate therapy directed toward correction of the cause of the obstruction. Such prompt action is essential for cure.

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Alterations of Electrolyte Metabolism During General Anesthesia

HARRY F. WEISBERG, M.D.

"I will put you to sleep" is what the anesthetist usually tells his patient prior to surgery. The sleeping state is a general lowering of all metabolic processes; but in anesthesia, the central nervous system is primarily depressed with variable effects upon the body's metabolism. The effects of anesthetics and/or narcotics is believed to be the result of dehydration of the nerve cells[1]. It is very difficult to evaluate the effects of different specific anesthetic agents on the various organs of the body.

WATER AND ELECTROYLTE ALTERATIONS

Many factors will affect the balance of water in the body. Anesthetic agents and narcotics vary in their effects but usually cause a stimulation or release of the antidiuretic hormone of the posterior pituitary gland, resulting in an antidiuresis[14, 15, 16]. Thus, limitation of the amount of water administered to a patient to less than normal amounts (e.g., on day of surgery) will summate with the anesthesia, resulting in an oliguria which is *not* the result of renal disease.

In anesthetized man, the blood volume is subject to many variations. Adriani[1] reports the blood volume to be reduced by hemorrhage, anemia, dehydration, shock, and exposure to cold, whereas it is increased by excitement, fever, muscular contractions, splenomegaly, liver disease, etc. Some surgeons request measurements of blood volume while fluids are being administered or while the patient is bleeding; and yet, despite such demands for exact data, there is usually only a vague guess at the patient's weight—a datum which is valuable in order to arrive at a proper blood volume determination. In addition to correcting for the central hematocrit and the amount of trapped plasma, corrections should be made for the sex and habitus of the patient. The values vary inversely with the amount of fat present; this is illustrated in Table I. The type of habitus can be readily determined by measuring the upper arm and scapular skinfolds with a caliper.

Alterations of electrolytes are usually judged by the changes in the serum

TABLE I VARIATION OF SOME BODY CONSTITUENTS WITH HABITUS*

	NORMAL HABITUS	LEAN (EXTREME)	OBESE (EXTREME)
Men (Adults):			
Blood Volume (ml/kg)**	76	87	65
Plasma Volume (ml/kg)**	45	53	37
Red Cell Mass (ml/kg)**	31	34	28
Water (% of Body Weight)	60	70	50
Fat (% of Body Weight)	18	4	32
Sp. Gr. (of Body)	1.062	1.091	1.034
Upper Arm Skinfold (mm)***	12	<7	>20
Scapular Skinfold (mm)***	16	<8	>33
Women (Adults):			
Blood Volume (ml/kg)**	66	77	55
Plasma Volume (ml/kg)**	43	52	34
Red Cell Mass (ml/kg)**	23	25	21
Water (% of Body Weight)	50	60	42
Fat (% of Body Weight)	32	18	42
Sp. Gr. (of Body)	1.034	1.062	1.015
Upper Arm Skinfold (mm)***	20	<12	>30
Scapular Skinfold (mm)***	16	<8	>33

*Modified from Weisberg.[40]

**Values may vary as much as ±10 per cent.

***Upper arm skinfold measured parallel to long axis of arm at *back of right* biceps, midway between tip of scapular acromial process and tip of elbow, with forearm flexed at 90° and arm hanging freely.

Scapular skinfold measured along line about 45° from horizontal level below tip of *right* scapula (See Brozek[9] and Brozek and Henschel[10]).

concentrations as reported from the laboratory. Often forgotten is the fact that concentration expresses the amount of solute present in a given amount of solvent or solution. Hyponatremia does not necessarily mean a loss of sodium from the extracellular fluid or other body compartments but may be the result of various physiological or pathological processes. Table II lists different

TABLE II CAUSES OF HYPONATREMIA*

I. *Delusional*:
  A. Laboratory error
  B. Displacement of Plasma Water:
    1. Hyperlipemia
    2. Hyperproteinemia
  C. Hyperosmolality of Plasma (non-electrolytes)

II. *Dilutional*:
  A. Excess Water:
    1. Exogenous ("acute")
    2. Endogenous ("chronic")
  B. Water shift:
    1. Hyperosmolality of ECF (non-electrolyte)
    2. Potassium depletion
    3. "Sick" cell syndrome

III. *Depletional*:
  A. Renal:
    1. Decreased reabsorption of sodium
    2. Osmotic diuresis
  B. G. I. tract
  C. Skin
  D. Lungs
  E. Paracentesis
  F. Hemorrhage (severe)

IV. *Distributional*:
  A. Sodium shift (into ICF)
  B. Sodium retention ("edema")
  C. Local accumulations

*Modified, with additions, from Weisberg[40].

categories of hyponatremia. Disregarding possible laboratory errors, the most important cause of a delusional low sodium is the displacement of plasma water (in which the sodium is dissolved) by elevated concentrations of protein or lipids[2]. The more common causes of hyponatremia are related to dilution[29] (excess water or shift of water), depletion or deficiency of sodium from the body, and distributional shifts (Table II).

The electrolyte imbalance seen in some surgical patients is frequently the result of chronic lack of intake of nutrients and/or lack of proper oral or parenteral preparation prior to surgery. Many surgeons refuse to believe that the electrolyte imbalance is present prior to the surgical procedure; if only "base-line" electrolytes had been ordered! The mechanism for sodium retention and potassium excretion postoperatively is probably related to the influence of aldosterone. Factors affecting the secretion of aldosterone are listed in Table III. It is reasonable for the physician to minimize the stimulation of aldosterone secretion by preventing or treating the occurrence of hyponatremia, reduced blood volume, and dehydration of the patient. Translated into a program of therapy, this means the administration of *proper amounts* of water and electrolytes prior to surgery and postoperatively[21, 27].

Sodium balance is intimately related to the renin-angiotensin system with the juxtaglomerular apparatus acting as the receptor site (Fig. 1). Changes of sodium levels of blood plasma are not very consistent, though sodium retention and hyponatremia occur, postoperatively[6, 27]. The blood level of potas-

TABLE III FACTORS AFFECTING SECRETION OF ALDOSTERONE*

INCREASED	DECREASED
Sodium restriction	Sodium load
Potassium load	Potassium restriction
Extracellular fluid ↓	Extracellular fluid ↑
Plasma (or blood) volume ↓	Plasma (or blood) volume ↑
Upright position	Lying position
Activity	Inactivity
Stimulation of thyroid-carotid arterial junction	Stimulation of vagus nerve
Acute constriction of inferior vena cava	Distension of right atrium
Decreased carotid artery pressure	Catecholamine in sodium-depleted patients
Stress: Traumatic (surgery, anesthesia)	Drugs: Spironolactone Amphenone B
Emotional	
ACTH	
Growth hormone	
Posterior pituitary	
Extract of diencephalon	
Angiotensin II	

*Modified from Weisberg[40].

sium usually falls with anesthesia but may be increased owing to even small amounts of anoxemia. The level of calcium is reported to fall with ether anesthesia and morphine. There are not too many studies regarding magnesium; reportedly it falls with ether anesthesia[1]. The changes in chloride concentration

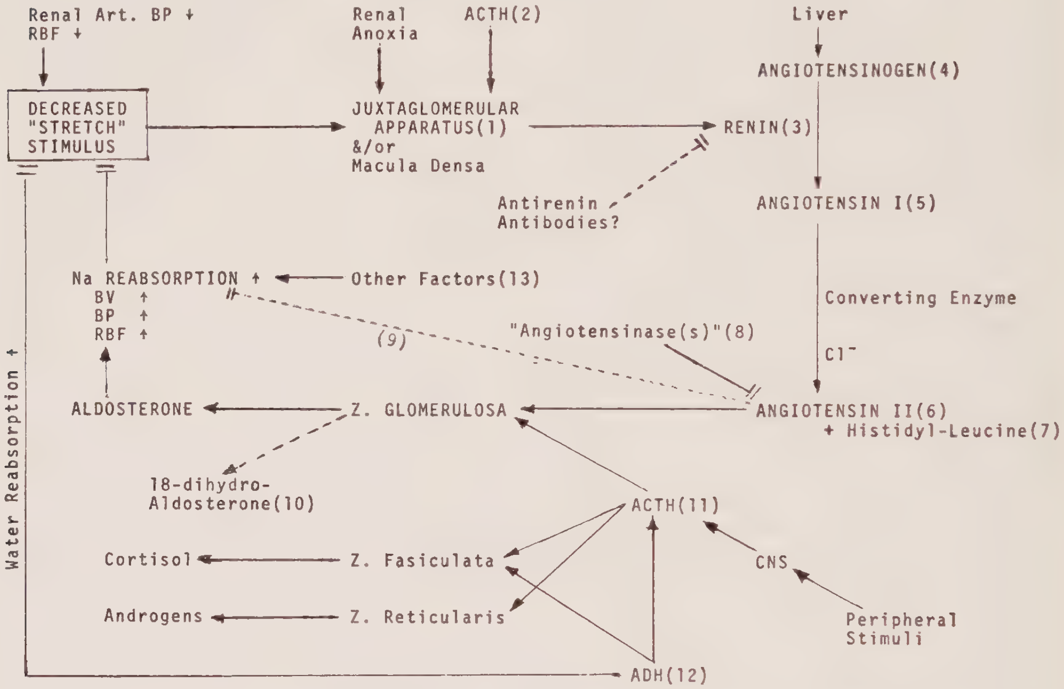

Fig. 1. Renin-Angiotensin System. Solid lines denote known functions; dotted lines, questioned. Arrows denote stimulation; double bar, "feedback" inhibition.

1. Juxtaglomerular apparatus, granularity increased; Macula densa, increased enzymes of hexose monophosphate shunt; increased pressor activity of kidney extracts.
2. ?Direct extra-adrenal action?
3. Acts as proteolytic enzyme.
4. With α2-globulin of plasma.
5. Inactive; a decapeptide.
6. Active; heat-stable; an octapeptide. Stimulates aldosterone production; also vasopressor and oxytocic substances; and (?) inhibits sodium transport.
7. A dipeptide.
8. Active at pH 7.5 in kidney and erythrocytes; ?also at pH 3?
9. In cirrhosis, angiotensin causes natruriesis?
10. ?Produced in malignant hypertension?
11. ACTH can initiate and/or support secretion of aldosterone. Provides TPNH for hydroxylation at Carbons 11, 17, 18 and 21?
12. Arginine vasopressin. Causes increased reabsorption of water by kidney; causes release of ACTH; and has a direct action on Z. fasciculata.
13. Other factors: (a) affecting sodium retention are cardiac output, GFR, venous pressure, Na intake, acid-base balance, plasma protein concentration, and other hormones (progesterone, estrogen, etc.); (b) low perfusion pressure causes increased Na reabsorption; and (c) ?kidney humoral substances per se?

are minor. Sulfate changes have not been recorded. Phosphate falls with most anesthetics but varies in extent, depending upon the conditions present. Lactate is increased with general anesthesia; the rise is greater in the presence of anoxemia.

Various formulas are available to calculate the osmotic pressure (osmolality) of the plasma when direct measurement by freezing point depression is unavailable. The following formula is from Holmes[22].

$$\text{mosM/kg} = 1.86\,[\text{Na}^+] + \frac{\text{BS}}{18} + \frac{\text{UN}}{2.8}$$

in which, $[\text{Na}^+]$ is the concentration of sodium expressed as milliequivalents per liter, and BS and UN are the concentrations of blood sugar and urea nitrogen, respectively, expressed as milligrams per 100 milliliters. The correlation with the measured freezing point value is good, providing no unusual solutes (e.g., drugs) are present in the plasma.

Hall and Reeser[20] demonstrated that passive hyperventilation is accompanied by a severe respiratory alkalosis and a low serum potassium. Andersen and Svane[3] have shown consistent changes in electrolyte concentrations induced by hyperventilation with a pump oxygenator for one hour, with resulting respiratory alkalosis. The pH rose with a concomitant fall in carbon dioxide tension and a fall in bicarbonate concentration. The concentration of potassium and phosphate fell. There was a small rise in calcium and a marked rise in lactic acid concentration. When the experiments were repeated (in animals), hyperventilation being accomplished with a carbon dioxide-oxygen mixture, hypercapnia was produced and the changes in the electrolytes were opposite to those listed previously. Recovery from hypocapnia was more rapid with the treatment of carbon dioxide-oxygen mixtures than when the subjects were allowed to recover spontaneously in room air. Oversized oxygenators in an extracorporeal system were used to produce extreme degrees of carbon dioxide washout in animals[4]. The usual response to acute hypocarbia occurred—decreased blood potassium and phosphate and increased blood lactate—but the depression of pCO_2 was more severe than reported in artificial ventilation.

RESPIRATORY EXCHANGE

Many factors affect the depth of anesthesia; some of these are peripheral resistance, blood pressure, cardiac output, sympathetic nervous stimulation, hemorrhage, liver glycogenolysis, ketone production, depression of enzyme systems and acidosis[1]. In addition to the anesthesia, the manipulative processes involved with the surgery, for instance, gastrointestinal suction and the amount of tissue trauma, must be added to the effects of prior disease of the patient to obtain the total stress and strain applied to the patient. Many patients will have a different response to the thought of surgery and will respond much differently with emotional overtones to the pre-anesthetic medication.

It is obvious that the surgeon and anesthetist are involved with the respiratory exchange of the patient before, during, and after the surgery. The conclusion is made that alveolar gas pCO_2 is equal to the arterial pCO_2, based upon the assumption that carbon dioxide gas in a single alveolus is equal to the carbon dioxide in the blood across the alveolar membrane. This assumption holds only for those subjects *with normal lungs.* Comroe et al.[13] report that such an estimate is erroneous if (a) there exists a large venous to arterial shunt, (b) pulmonary vascular occlusion is present, (c) there is a serious maldistribution of blood and/or alveolar gas, (d) rapid and shallow breathing is present, or (e) Diamox is used in therapy. Even if the minute volume of alveolar ventilation is normal or increased, the venous blood will not be oxygenated in the lungs (a) if the ventilation is not uniform, (b) if the pulmonary capillary blood flow is not uniform, or (c) if the diffusing capacity of the lung is decreased[13].

An elevation of arterial pCO_2 denotes that the whole lung or a major portion is hypoventilated; a decreased arterial pCO_2 denotes hyperventilation of a major portion or the whole lung. A normal arterial pCO_2 does *not* exclude the presence of pulmonary disease, i.e., the alveolar ventilatory effort is able to match the demands of body metabolism. This may be seen in subjects with a reduction of pulmonary tissue due to a pneumonectomy or to a pulmonary embolus; in patients with pulmonary vascular disease; and in subjects with impaired diffusion. There may be severe pulmonary disability and dyspnea due to the mechanical effort to achieve a normal pCO_2. The arterial pO_2 is not as good a guide to alveolar ventilation since it is influenced by an impairment of alveolar-capillary diffusion (whereas the pCO_2 is not)[13]. However, the tissue pCO_2 (e.g., of brain) may vary independently of the arterial levels[28].

Stephen *et al.*[35] discussed the anomalies of ventilation during anesthesia. These anomalies are irregularities which may occur and, therefore, embarrass the ventilation of the patient during surgery. Other factors may affect the patient during anesthesia. The preoperative condition of the patient can affect the pulmonary ventilation and limit markedly the pulmonary reserve, the margin of error allowable to the anesthesiologist during the anesthesia. Such conditions as intrinsic asthma, pulmonary tuberculosis, bronchiectasis and pulmonary fibrosis or emphysema can precipitate profound disturbances in the pulmonary ventilation-blood flow ratios[35].

Preoperative medication can affect the sensitivity of the respiratory center and will also have other effects on the biochemical changes within the body. Tomlin et al[38] reported a group of 24 patients premedicated with atropine to show a mean oxygen saturation of 93.4 per cent, a value significantly lower than the 96.0 per cent obtained in a control group of 24 patients who were not given atropine. The atropine group had a mean oxygen tension 15.4 mm Hg less than the control group. They[38] concluded that atropine can cause significant hypoxemia and that its routine use as a pre-anesthetic medication is no longer justified. However, Gardiner and Palmer[18] studied thirty-one patients given atropine or papaverine and scopolamine. They did not find a change in arterial oxygen saturation or tension when both values were measured directly. Taylor *et al.*[37] studied six young adults who had minor surgery (uterine curettage). Papaverine and atropine were given one hour prior to the anesthesia with halothane in oxygen. Though the oxygen saturation did not change, the arterial pCO_2 at the end of twenty minutes of anesthesia was 55 mm Hg in contrast to the control value of 40 mm Hg; the corresponding pH values were 7.276 versus control 7.408. Thus, even light anesthesia has a depressive effect upon respiration.

The analgesic drugs for pain and the anesthetic *per se* have a depressant effect upon respiratory ventilation. It is unfortunate that this is forgotten by many physicians. In addition, the muscle relaxing drugs which are necessary to paralyze the diaphragmatic and intercostal muscles for certain surgical procedures do affect the respiratory efforts of the patient. Technical factors related to the surgery will affect the diffusion of gases across the pulmonary tree; for instance, partial immobilization of the diaphragm by abdominal packs will limit the excursion of the lungs. Thoracotomy or other procedures in the chest cavity will affect the respiratory exchange. The technical factors inherent in the anesthesia administration, either by a face mask (which can increase the mechanical dead space) or the more complex gas machines and mechanical ventilators, are not within the scope of this presentation.

One aspect that must bear emphasis is the operative position of the patient. This has been shown to be important in manual artificial respiration; for instance, the Schafer method popular in Red Cross and Boy Scout courses in first aid, will only allow for passive respiration by pressure on the patient in the prone position. This method is inadequate, especially in patients in need of artificial respiration. The Schafer method promulgated in 1903 is similar to the old technique of resuscitation of individuals from drowning, popular in Europe in 1812, utilizing a trotting horse. The victim was placed athwart the back of a horse, forcing out the air; this also was a passive expiratory excursion of the lung. The Silvester technique (1858) utilized the patient in the supine position with an arm-lift chest-pressure. This gave twice as much respiratory exchange, utilizing the inspiration and expiration phases. The Silvester method was not satisfactory, because the patient had his airway obstructed due to the supine position. In 1932, the Holger-Nielsen prone arm-lift back-pressure technique was instituted, and this gave a large amount of respiratory excursion. However, because of the position of the head and neck this, too, may give rise to serious mechanical obstruction of the airway. Various other methods of artificial resuscitation have been utilized[12] but are now being replaced by the mouth to mouth or mouth to nose insufflation, also called mouth to mouth breathing or expired air inflation[13]. Patients undergoing abdominal operations in the supine position, if allowed to breathe without assistance by the anesthetist, will always have an increase in carbon dioxide tension whereas vigorous assisted respiration will keep the carbon dioxide tension within normal limits. It is impossible to deliver proper ventilation to a patient in the Trendelenburg position, the rise in carbon dioxide tension being proportional to the angle of tilt. In cases that are being operated upon in the thoracic area, a lateral position, especially if the table is broken, causes a respiratory acidosis. Even the lithotomy position will cause a reduction in vital respiratory capacity, especially if the head is much lower than the hips.

In addition to the position upon the

operating room table, any position or condition that favors elevation of the diaphragm predisposes to atelectasis[24]. On the other hand, the full inspiratory position such as seen in the thorax of some patients with chronic pulmonary emphysema results in a diaphragm which is low and flat[13]. Contraction of such a diaphragm is either ineffective or may actually pull the thorax in during inspiration. The position of Jesus Christ upon the Cross with arms extended is believed by Bucklin[11] to have contributed to spasm of the thoracic muscles, which were unable to be utilized in respiration. These factors may well have contributed to the reported death of Christ after being suspended for approximately three hours. In addition to the theories concerning the cause of death as pericardial infusion or congestive heart failure or hydro-hemothorax, the impaired respiration with respiratory acidosis is probably another possibility.

The assistance or control of respiratory exchange by the help of the anesthetist may lead to hypoventilation or hyperventilation. These changes may be intentional (iatrogenic) or by inadequate assistance. "Controlled" respiration is a term used to describe deliberate hyperventilation on the part of the anesthetist to achieve a possible fatigue of the Hering-Breuer reflexes[33]. The hyperventilation will cause a relative alkalosis with the blood being rich in oxygen and decreased in carbon dioxide. The hypoxic drive to the carotid body is excluded; therefore, the addition of drugs or other factors that will depress the respiratory center is not advisable in this procedure[33]. At the end stages of controlled respiration a metabolic acidosis may occur[31]. The changes may be very rapid. After hyperventilation for three minutes in twelve subjects, the pCO_2 changed from 39.8 to 17.2 mm Hg and the pH changed from 7.4 to 7.6[34]. Table IV summarizes the effect of respiratory ventilation on pH, pCO_2 and oxygen saturation.

TABLE IV EFFECT OF RESPIRATORY VENTILATION ON pH, pCO_2 AND O_2 SATURATION

	pH	pCO_2 (mmHg)	O_2 Sat. (%)
Hypoventilation			
Room air	↓	↑	↓
With oxygen	↓	↑	N
Hyperventilation			
Room air	↑	↓	N* (↑)
With oxygen	↑	↓	N* (↑)

*In (arterial) blood, hemoglobin almost completely saturated with oxygen at PO_2 present during quiet breathing of room air. Breathing oxygen raises PO_2 (with increased solution of O_2 in plasma), but does not increase saturation (oxygenation) of hemoglobin. Hyperventilation will raise oxygen saturation if alveoli were hypoventilated at beginning.

Anesthetists deliberately hyperventilate their subjects to achieve a respiratory alkalosis for the following reasons: (a) it protects against respiratory acidosis which can result from anesthesia (causing respiratory depression); (b) the relatively high oxygen content of the blood allows the use of higher concentrations of relatively weaker anesthetic agents such as nitrous oxide (without the hazard of anoxemia from hypoventilation); (c) muscle relaxants are used in smaller doses since the hyperventilation enhances the action of curare drugs, and (d) it can be used in conditions in which metabolic acidosis is produced, for example, extracorporeal perfusion and temporary circulatory occlusion of certain parts of the body.

"Apneic oxygenation" is a term used for "diffusion respiration" and has been called "apneic diffusion oxygenation." These terms refer to the insufflation of 100 per cent oxygen into the lungs of the patient. The action of the heartbeat agitates the alveolar area to allow diffusion of oxygen into the pulmonary circulation—the so-called "heart-hemoglobin pump." Such insufflation of oxygen will allow maintenance of the patient without active respiratory movements for up to thirty minutes[17, 33]. An apnea for five minutes results in the change of pH from 7.46 to 7.26, the pCO_2 from 33 to 73 mm of Hg, and the CO_2 content from 25.5 to 34.2 mM/L[19]. However, with circulatory arrest at low temperatures the pH does not vary very much, presumably because the deoxygenated blood can carry more hydrogen from the organic acids produced by metabolic activity. Upon restarting of the circulation and oxygenation, however, a metabolic acidosis was noted[39]. Stephen and Talton[36] have reported a period of hypoxemia postoperatively when patients were breathing room air; this was not accompanied by a respiratory acidosis. Bergofsky *et al.*[5] report that acute hypoxia is an independent stimulus causing pulmonary vascular constriction; at the same time acute hypercapnia (with hydrogen ion increase) will also give rise to pulmonary vascular constriction. Roberts *et al.*[32] have summarized their findings in early respiratory alkalosis and in the late stages of respiratory alkalosis due to excess pulmonary ventilation in which a metabolic acidosis will be produced.

ARTERIAL VERSUS VENOUS BLOOD

A controversy has existed between various investigators concerning the use of venous blood rather than arterial blood for the evaluation of acid-base imbalance. Nunn[30] states, "If the blood is venous, the metabolic state will be substantially the same as that of the arterial blood, but the respiratory state will differ from that of the arterial blood depending upon the tissues through which the blood has passed." Woolmer[41] points out that the pCO_2 of arterial blood is a constant 6 mm Hg below that of mixed venous blood. The study by Brooks and Wynn[7] concludes that venous blood can be used under certain conditions. This is exemplified in Table V. The conditions which they call for are that the patient should be at bed rest and should be warm, the skin temperature over the dorsum of the hand measured by a mercury thermometer in contact with the skin being at least 35° C (95° F), and that the blood should be obtained without stasis from a superficial vein of the hand. Broome and Holt[8] point out that stasis *per se* is not as significant as the avoidance of forearm exercise when obtaining a venous blood specimen; this study was in reference to electrolytes, the source of error being the exercise rather than stasis for 2 minutes. Table V illustrates that the ambulatory position results in higher values in the venous blood specimens in comparison to the arterial specimens. A patient who is ill and has warm extremities does not need to have the arm warmed in order to obtain "arterialized" blood. This is especially so in those patients undergoing general anesthesia where the differences were

Table V Mean Arteriovenous Differences in Plasma pH, CO_2 Content and pCO_2*

No. Subjects	Diagnosis	Condition	Mean Skin Temperature °C	Mean Skin Temperature °F	"Heated" Vein** pH	"Heated" Vein** CO_2CT (mM/L)	"Heated" Vein** pCO_2 (mm Hg)	"Unheated" Vein pH	"Unheated" Vein CO_2CT (mM/L)	"Unheated" Vein pCO_2 (mm Hg)
1 (4X)	Advanced emphysema Cor pulmonale	Ambulatory	34.2	93.5				+0.063†	−3.7	−16.9
5	Convalescent	Ambulatory	38.2	100.7	+0.018	−1.3	−3.5	+0.061	−2.4	− 9.6
9	Convalescent (4) Mod. to severely ill (5)	Bed rest	38.4	102.0	+0.002	−0.4	−0.8	+0.045	−1.2	− 6.7
7	Severely ill (Extremities warm)	Bed rest	35.2	95.3				+0.010	(?)0.2	− 1.2
10	General anesthesia	"O.R."	35.4	95.7				+0.002	(?)0.3	(?) 1.1

*Modified from Brooks and Wynn[7]. Artery: brachial or femoral. Vein: back of hand or wrist; no stasis.

**Two electric pads for fifteen minutes.

†A-V: Therefore, − and + represent value for vein higher and lower, respectively, than for artery.

practically negligible. In a larger series of 200 patients studied by Little *et al.*[26], blood was taken from the artery of one arm and from the vein of another arm while the patients were under anesthesia. Fifty-seven had normal spontaneous respirations, twenty-four were assisted manually and 119 were assisted by mechanical ventilators. Under these conditions, with anesthesia varying from 10-190 minutes, the values for arterial pH were 7.37 ± 0.11 and the venous pH was 7.36 ± 0.11; the correlation being 0.985. The values for pCO_2 were arterial 40.09 ± 13.85 and venous 42.20 ± 13.55 mm Hg; the correlation was 0.978. The major objection to arterial blood sampling has been the difficulty of the house staff to obtain a brachial or femoral arterial puncture. Knudsen and Hansen[25] report that arterialized blood from the earlobe has a better correlation with arterial blood than does blood from the fingertip; the question arises whether or not the cooling of the finger is sufficient to explain this. Jennett and Moody[23] suggest sampling of arterial blood from the field of operation with microcapillaries as an easy source of obtaining arterial blood specimens, if necessary.

In our experience, measurements of venous blood samples are satisfactory since they are based upon venous normals, and for the same "conditions" the changes are usually reflected in the patient. It is not necessary to obtain arterial blood unless special research studies are being undertaken or if pulmonary-cardiac studies for diagnosis are necessary.

SUMMARY

1. The changes in metabolism of electrolytes during anesthesia are difficult to evaluate because of the various intangibles present prior to anesthesia, during the anesthesia and during the

surgical procedure. The medical background of the patient and the type of pre-anesthetic medication also affect electrolyte balance.

2. Changes in electrolyte concentration are mediated primarily by aldosterone, which is controlled by the reninangiotensin system and the status of acid-base existing in the patient.

3. Most instances of electrolyte imbalance prior to and during the surgery can be prevented by proper knowledge and administration of fluids and electrolytes to the patient as part of the presurgical workup.

4. Because of the popularity of "controlled" respiration and "diffusion respiration," better monitoring of the acid-base status of the surgical patient is indicated. Under most circumstances, venous blood can be used and the methods performed in a routine laboratory; under special circumstances, such as research studies and investigation of cardio-pulmonary disorders, arterial specimens may be indicated and are usually done by special units.

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The Serum Electrolytes in Pneumonia and other Infectious Diseases

HERBERT DERMAN, M.D., and DAVID S. GERBARG, M.D.

Since 1850, it has been known that chlorides almost disappear from the urine in lobar pneumonia. Redtenbacher's and Beale's original observations were eventually followed in the 1890s by Hutchinson[7], intensively studied in the 1920s by a group at the University of Pennsylvania, and last reviewed in the literature by Sunderman in 1951[21]. All workers[3, 8, 16, 18] agreed that urinary chlorides were depressed, but no information explained the mechanism.

Initially, the pursuit of an understanding of lobar pneumonia had to await the discovery of its etiology in the 1880's by Pasteur, Sternberg, and Frankel[9]. Clinical science was then in its infancy, and the chemical technics to make the basic measurements needed to elaborate the mechanism of chloride retention only became available in the 1920's. Before the fruits of these investigations could ripen, Avery *et al.*[10] developed specific antiserum therapy for lobar pneumonia. The laboratory emphasis promptly shifted from chemistry and physiology to bacteriology and immunology. The final closing of the door on the "great chloride mystery" occurred with the advent of sulfonamides and penicillin. Dreaded pneumonia had been conquered, and cases all but disappeared from the wards of the teaching hospitals.

There may no longer be any diagnostic or therapeutic necessity to study urinary chlorides in lobar pneumonia. However, in understanding physiologic reactions in infection, the solution of the "great chloride mystery" has the same promise today that it had in 1850. Therefore, it may serve a useful purpose to review this subject again. With the investigative tools now available, it should be possible to delineate the mechanisms by which the body simultaneously lowers the serum and urine chloride levels in lobar pneumonia[24, 25].

How Great is the Diminution of Serum and Urine Chlorides in Lobar Pneumonia?

The normal range of urine chloride excretion per twenty-four hours is 6 to 9 gm.[26] This value is directly related to the chloride intake. Prior to the crisis in lobar pneumonia, the twenty-four-hour urinary excretion of chloride is less than 1 gram per day[7] and frequently less than 0.1 gm. per day. In lobar pneumonia, the serum chloride level tends to be 10 per cent lower than normal[23].

What Happens to Serum and Urine Chlorides after the Pneumonic Crisis?

The urinary excretion rises abruptly to as much as 21 gm. of sodium chloride per day[7]. The serum level returns to normal before the urinary level stabilizes at normal[11].

Are the Serum and Urine Chloride Levels Related to Fever?

In lobar pneumonia the degree of urinary chloride retention is unrelated to the height or duration of fever[7]. In other febrile states, urine chloride is slightly diminished except in malaria, where it is increased[7].

Experimental work by Binger, Christie, Davis, and Hiller[2] in the dog demonstrated that serum chloride depression occurs with pneumococcal infection, but not with anoxemia, tissue destruction, anaphylactic shock, experimental leucocytosis, or fever alone, when these are not related to pulmonary pneumococcal infection.

What is the Urine Chloride Level in Other Lung Diseases?

Urinary chlorides are never as low in bronchopneumonia or other lung diseases as in lobar pneumonia[7, 19].

Is There an Impairment of Intestinal Absorption of Chloride in Lobar Pneumonia?

Both Röhmann and Terrary[27], in the late 19th century, established that ingested sodium chloride is completely absorbed from the gastrointestinal tract in lobar pneumonia.

Can the Hypochloremia of Pneumonia be Affected by Administration of Desoxycorticosterone Acetate?

Newburger[14] reported no effect on the concentration of serum chloride from 10 mg. of *DOCA* per day in four patients with lobar pneumonia.

Is There a Relationship between Chlorides and other Urine Constituents in Lobar Pneumonia?

Holten[6] has reported that increased amounts of organic acids are present when the chloride is depressed, and Austin and Sunderman[1] have shown a severe reduction in the excretion of fixed base. In addition, these workers showed excessive excretion of sulfate and phosphate in the urine of precritical pneumonia patients to compensate for the chloride depression. This results in a normal excretion of total anion. The high sulfate excretion is proportional to high nitrogen excretion and is probably correlated with tissue catabolism.

Is There any other Significant Route of Excretion of Chlorides from the Body in Lobar Pneumonia?

The fecal content of sodium chloride is practically nil[7]. The skin loss of sodium chloride is not sufficiently increased in lobar pneumonia to account for the missing chloride[17]. The sputum in lobar pneumonia contains 0.3 to 0.5 gm. sodium chloride per twenty-four hours[7], which is insufficient to account for the chloride mystery.

Where else can Chloride Hide?

It has been documented repeatedly since 1869 that weight loss is constant in pneumonia, and therefore, simple dilution by retained water is not a satisfactory explanation of the hypochloremia of pneumonia. Sunderman[22] has shown that total base is decreased; sodium is decreased; calcium is decreased; but potassium is increased. Present concepts do not differ significantly from those of Peabody[15] who, in 1913, believed that the retained chlorides diffusely spread in the viscera, muscles and tissue of the body.

Is the Urinary Excretion of Chloride Related to the Concentration of Serum Chloride in Lobar Pneumonia?

Prior to the crisis, they are both decreased in the patient who is not receiving a large supplemental salt intake. The correlation does not hold in the patient receiving salt. With respect to the cations, there is a general correlation between urinary and serum levels. Sodium and calcium are low in both serum and urine; potassium is increased in both; magnesium shows no consistent change.

Is the Urine Chloride Excretion Related to the Intake of Chlorides during Pneumonia?

With normal dietary intake of chloride or with moderate supplementation, there is no effect on chloride excretion in the urine during pneumonia[6]. The net retention of chlorides in lobar pneumonia is from 8 to 20 gm.[7] in the pre-crisis period. When large amounts of chloride are given experimentally, the urinary excretion of chloride increases but always remains well below the intake level[4]. As much as 65 gm. of sodium chloride have been retained by patients with lobar pneumonia who have been fed salt by mouth[4, 5].

Some interesting balance studies were reported in pnuemonia patients by Sunderman in 1929[25]. Patients with pneumonia were fed excessive quantities of salt and were compared with untreated cases for control. During the pre-critical period, the control pneumonia patients were in negative balance, excreting more chloride than ingested, despite the fact that both serum and urine chlorides were low. The salt-treated patients were in positive balance, although they excreted considerable chloride in the urine. After the crisis, untreated patients excreted increased chloride in the urine, but, nevertheless, the chloride balance became positive. These paradoxical observations lead us to the next question.

Is the Chloride Retention in the Body Secondary to Water Retention?

The amount of salt retained in lobar pneumonia may be of the order of 20 gm.[6, 7] This would require 2.5 l. of water to make it an isotonic fluid, and this degree of edema is not described in lobar pneumonia. Furthermore, if salt is given to patients with lobar pneumonia they retain it[4, 5], as previously noted. Some degree of fluid retention in lobar pneumonia was recorded by Maver and Schwartz[13] and by Sunderman[22]. Analyses by Sunderman[20, 23] indicated that in pneumonia patients receiving only a small daily intake of chloride, the mean concentration of chloride in body water remained unchanged from normal, or decreased during the pre-critical period. After the crisis, the mean concentration increased. In those patients receiving a large salt intake, the mean concentration of chloride in body water increases before the crisis. These observations suggest that the basic problem in pneumonia is not simple chloride retention but a diminished capacity to conserve chloride on a low intake, and a diminished capacity to excrete chloride on a high intake. McIntosh and Reimann[12] showed renal function was normal in pneumonia.

The phenomena of electrolyte control in lobar pneumonia are described, but these remain as obscure in pathogenesis today as when first noted 114 years ago. Their explanation to aid in the management of pneumonia is no longer necessary, but they still offer themselves as a natural experiment in chloride metabolism which must be solvable in this era of sophisticated methods of research.

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Fluid and Electrolyte Metabolism in Liver Disease*

CARROLL M. LEEVY, M.D.

Numerous studies have been conducted on the occurrence, mechanism, and treatment of fluid and electrolyte imbalance in liver disease. The normal liver is of key importance in regulating water and electrolyte metabolism, since it (a) receives, temporarily stores, and regulates release of ingested water and salts; (b) produces albumin, the chief determinant of serum colloidal osmotic pressure; and (c) is partially responsible for the conjugation and excretion of antidiuretic hormone, aldosterone and corticosteroids. Fluid and electrolyte alterations in liver disease may occur because of inadequate or excess intake of water and salts; the presence of hepatic, venous, or lymphatic blockade, which interferes with transit across the hepatic and splanchnic bed[1]; and deficient production of albumin or decreased conjugation and excretion of water and electrolyte regulating hormones[2–4]. Of equal importance is the fact that severe liver injury is often accompanied by kidney dysfunction, which interferes with renal control of electrolyte metabolism. Concomitant liver and kidney necrosis may be present in conditions such as carbon tetrachloride intoxication. Acute renal tubular necrosis may occur in liver disease associated with shock, and for reasons as yet unknown, in the absence of significant morphological changes, there may be reduction in renal blood flow, decreased glomerular filtration, and medullary shunting[5, 6, 7].

Liver failure is often accompanied by an increase in total body water, an increase in exchangeable sodium, a decrease in exchangeable potassium, an increase in plasma volume, and acid base disturbances[8, 9, 10]. These abnormalities are exaggerated when there is associated renal failure (Figure 1). A variety of other changes, such as protein, magnesium, zinc, or cadmium deficiency are frequently present in severe liver disease.

CLINICAL RECOGNITION

Normal subjects excrete eighty to ninety per cent of infused or ingested water in the urine during the succeeding four to six hours. A reduction in serum osmolality imposed by a water load inhibits release of antidiuretic hormone and permits renal excretion of dilute urine. Slight degrees of water retention are noted in acute hepatitis and fatty liver, while marked retention usually occurs in cirrhosis with ascites[11].

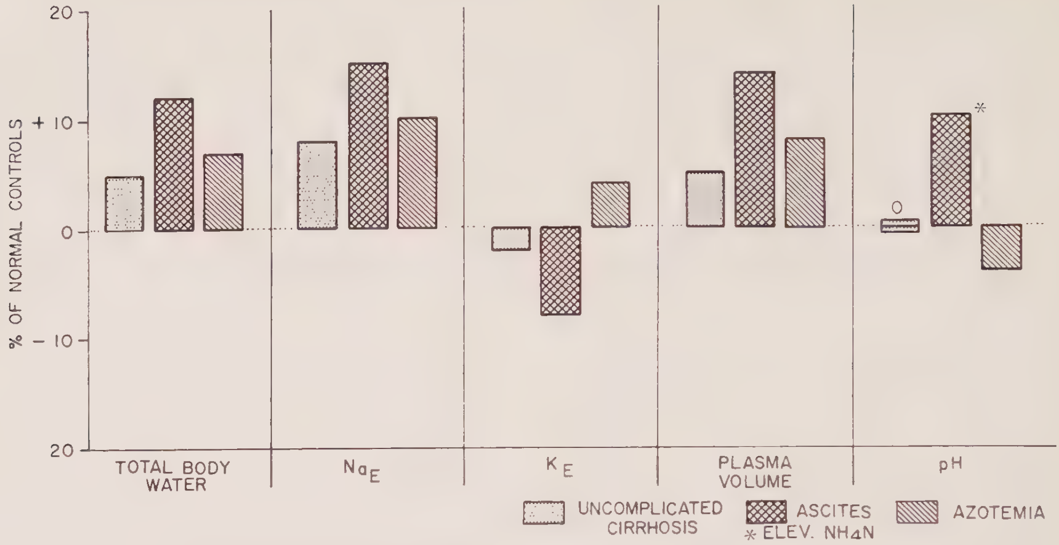

Fig. 1. Summation of data recorded in literature on water and electrolyte imbalance in cirrhosis.

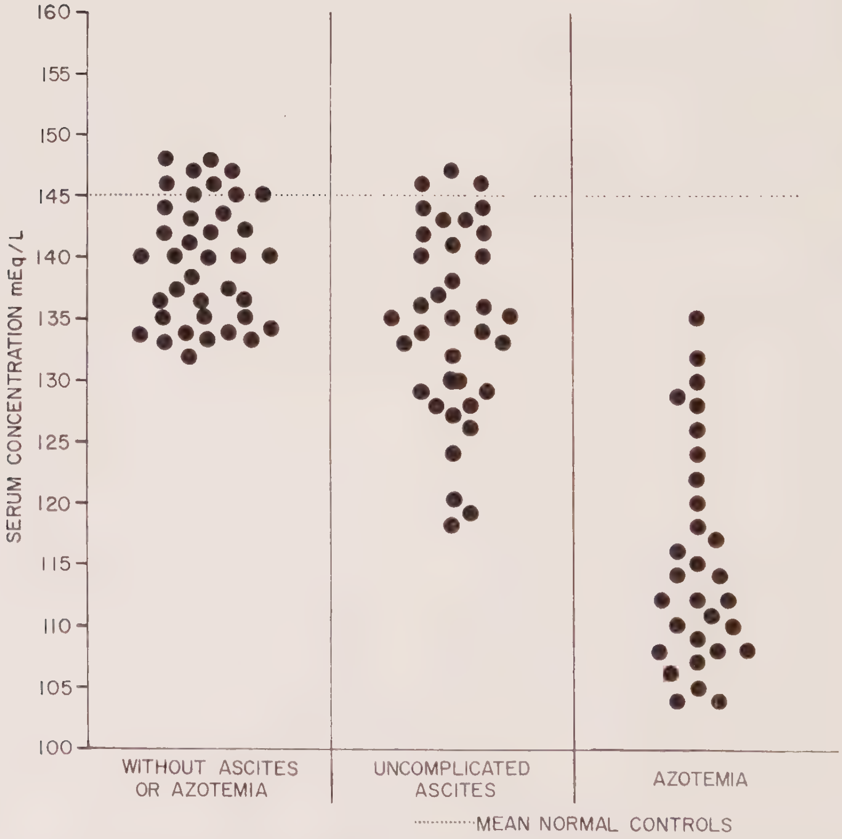

Fig. 2. Serum sodium concentration in cirrhosis of the alcoholic. Values represent admission levels without regard to prior receipt of diuretics.

In these instances, hemodilution often persists with maintained antidiuretic hormone activity and prolonged reduction in osmolality of the serum. Improvement in hepatic reserve in patients with reversible liver injury is frequently followed by a spontaneous diuresis and a restoration of normal water tolerance.

Despite the almost universal presence of an increase in total exchangeable sodium in liver disease, there is frequently a reduction in the concentration of serum sodium. Hyponatremia is most marked in the presence of ascites and azotemia (Fig. 2). Encountered changes in serum sodium usually follow the use of diuretic therapy for peripheral edema or ascites, but they may occur spontaneously. Acute reduction of serum sodium represents a major hazard in the patient with liver disease, since it is frequently accompanied by significant degrees of hypotension which may precipitate renal failure or hepatic encephalopathy. Simultaneous measurements of serum sodium and plasma volume permit separation of patients with sodium depletion into a group with normal concentrations of sodium and a group with decreased concentrations of sodium and decreased plasma volume (Fig. 3). Also, use of such combined

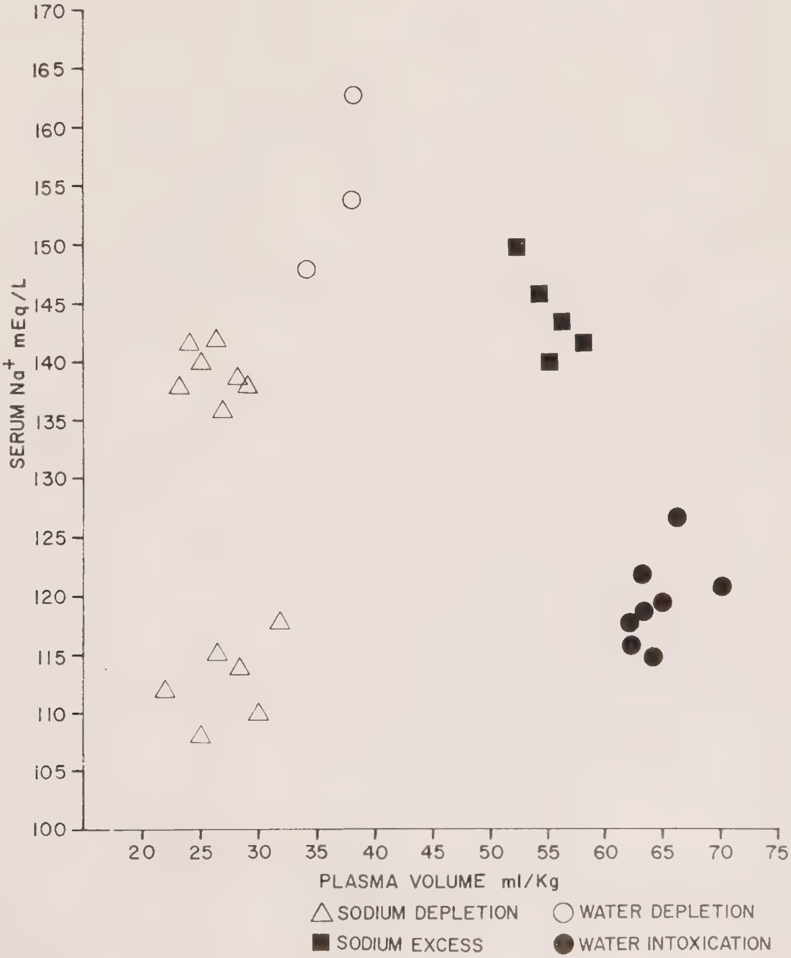

Fig. 3. Relationship of serum sodium concentration and plasma volume in cirrhosis of the alcoholic.

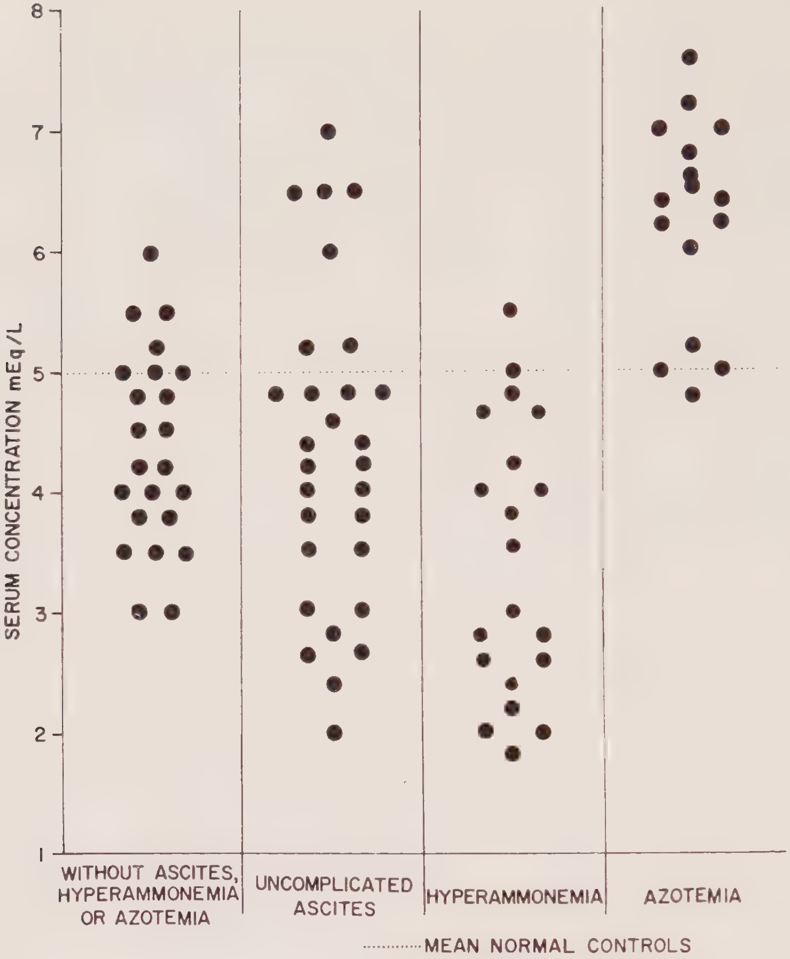

Fig. 4. Serum potassium concentration in cirrhosis of the alcoholic. Values represent admission levels without regard to prior receipt of diuretics.

measurements facilitates identification of hyponatremia which results from an expanded plasma volume[12].

Potassium deficits often seen in untreated patients with cirrhosis and ascites are also accentuated by use of diuretic agents which produce a kaluresis (Fig. 4). Occasionally a marked decrease in serum potassium will occur in a patient receiving diuretic drugs, despite the liberal use of oral potassium supplements. Hypokalemia is of particular importance, since it may lead to ammonium intoxication or acid-base disturbances and may interfere with protein and nucleic acid metabolism[13]. Hyperkalemia, which produces equally devastating effects on myocardial and cerebral metabolism, is often noted in the patient with liver disease and azotemia, or following the administration of supplemental potassium or the use of aldosterone inhibitors.

Elevation of the blood ammonia is often present in liver disease associated with fluid and electrolyte imbalance. It may result from either hepatic cell dysfunction or vascular shunts which interfere with ammonia fixation by the liver. Hyperammonemia is reflected in electroencephalographic changes and a variety of neuropsychiatric abnormalities. Ammonium intoxication is characterized by respiratory alkalosis; less frequently, particularly in the presence of azotemia, it is accompanied by a reduction in pH due to the accumulation of fixed acids. Approximately 10 per cent of patients with hepatic coma do not have an increase in blood ammonia. Theroretically, such patients may still have ammonium intoxication, since changes in pH facilitate transfer of ammonia ions across the cerebrovascular barrier. Clinical studies indicate, however, that this mechanism is not important in man and suggest that biogenic amines and other substances may be responsible for observed mental alterations[14].

Changes in effective circulating volume are perhaps the most important single abnormality of fluid and electrolyte metabolism in liver disease. Unfortunately, it is not possible to obtain a valid measure of this parameter in either normal subjects or sick patients. The closest approximation is currently provided by estimation of plasma volume or red blood cell volume. Serial investigations of plasma volume, as related to body weight,

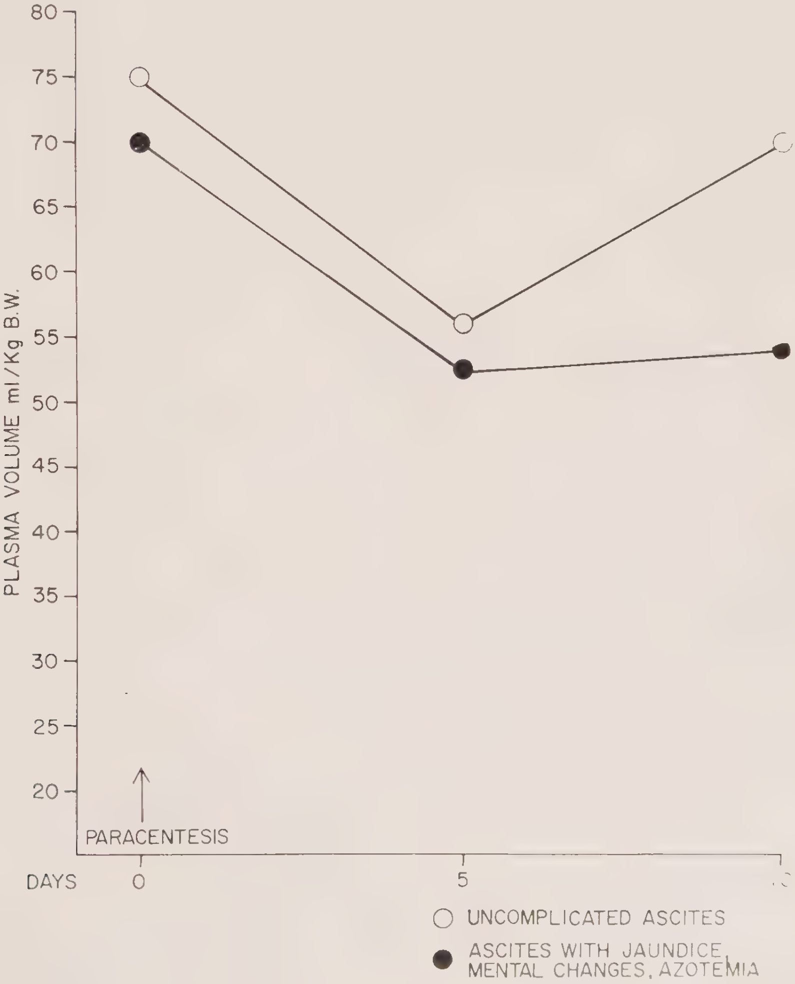

Fig. 5. Influence of paracentesis on estimated plasma value in cirrhosis of the alcoholic.

show a significant difference between patients with cirrhosis and ascites who are responsive and refractory to diuretic therapy. This difference is more apparent if serial studies are obtained during different phases of progressive liver failure. Diuresis evokes a much more significant reduction in plasma volume in patients responsive to a diuretic regimen; moreover, compensation for reduction in plasma volume following paracentesis is significantly different in the patient with and without evidence of liver failure. The patient with cirrhosis and ascites without evidence of liver failure has rapid restoration of plasma volume to pretherapy levels, whereas, those with hepatic insufficiency may exhibit negligible or greatly delayed restoration of prior plasma volume (Fig. 5).

THERAPEUTIC ASPECTS

Therapeutic response and overall prognosis in patients with fluid and electrolyte imbalance in liver disease depend upon improvement in hepatic functional reserve. Attention should be directed to control of ascites, mental aberrations, renal dysfunction, and specific alterations in serum electrolytes. Ascites must

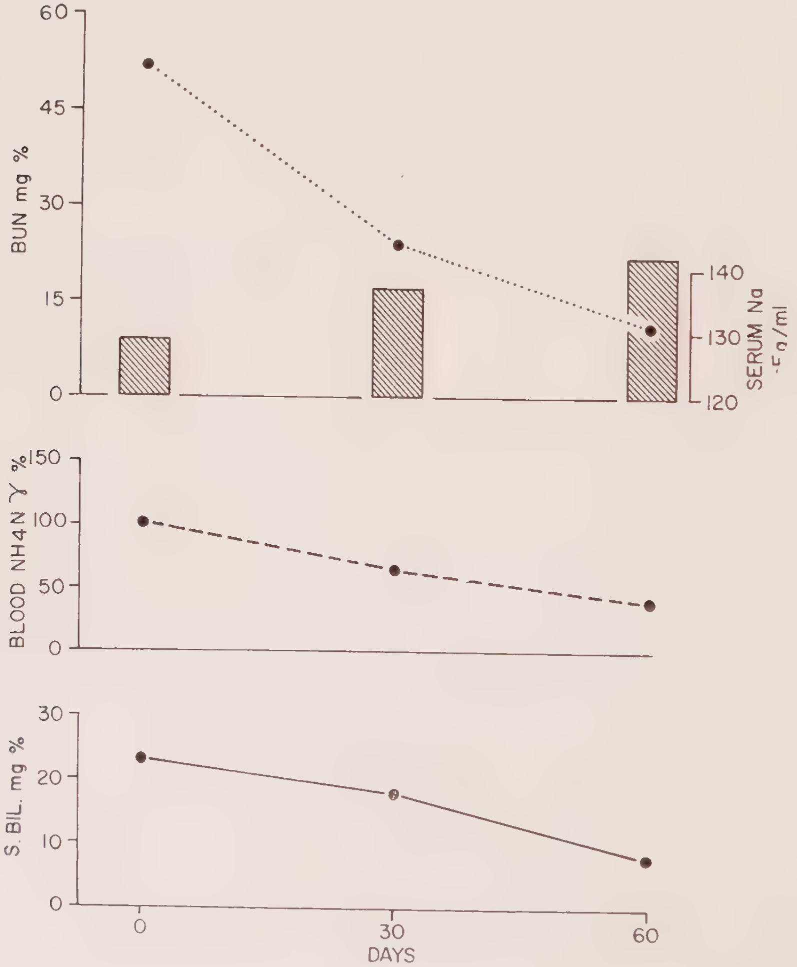

Fig. 6. Reversible azotemia in cirrhosis of the alcoholic with ascites.

be treated with sodium restriction and diuretics[15], avoiding, if possible, precipitation of hyponatremia, hypokalemia, and hyperammonemia. The patient with hepatic encephalopathy and elevated blood ammonia should receive potassium supplements to correct hypokalemia, and protein restriction and antibiotics to suppress ammonium production[16]. Little can be done for most instances of renal dysfunction and azotemia which complicate hepatic disease, though modification of water intake and use of plasma expanders may be helpful in selected patients. Improvement in hepatic reserve is usually associated with recovery from renal failure. This is illustrated in Figure 6, where ingestion of a nutritious diet by an alcoholic patient with hepatic and renal abnormalities was followed by simultaneous decrease in serum bilirubin, blood ammonia and blood urea, and an increase in the concentration of serum sodium.

It is important to assess periodically the functional reserve of the liver and to evaluate its regenerative potential in management of water and electrolyte imbalance in liver disease. A variety of parameters are available for these purposes. A study of plasma clearance of indocyanine green, which is entirely removed from the circulation by the liver, provides the best available overall index to uptake, storage and excretory capacity of the severely damaged liver[17]. Following administration of 0.5 mg. per kg. of indocyanine green to a patient with advanced cirrhosis, the percentage disappearance rate is markedly reduced and half-time increased, as compared with events in a normal patient (Fig. 7). Moreover, while removal capacity of *ICG* for 0.5 mg. and 5.0 mg. per kg. is approximately

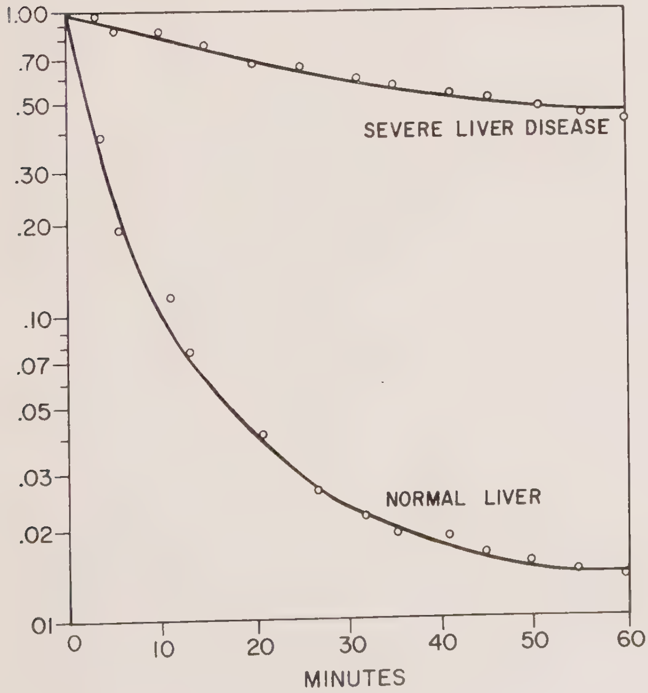

Fig. 7. Evaluation of hepatic functional status from plasma decay of indocyanine green.

the same in subjects with a normal liver, patients with mild liver disease exhibit a distinct reduction in removal capacity for the higher dosage[18]. The dichromatic ear densitometer, which was devised to estimate cardiac output using *ICG*, permits a study of hepatic clearance of this dye without the need for repeated blood punctures. The densitometer* provides a reliable index to arterial levels of the dye[19] and is not affected by blood volume, oxygen changes and skin pigment. The use of *ICG* and ear densitometry for serial quantitation of hepatic function is advocated in patients with liver disease accompanied by fluid and electrolyte imbalance.

Evaluation of hepatic regenerative potential has been made possible by development of an *in vitro* method to study hepatic *DNA* synthesis in percutaneous biopsies. Biopsies have been incubated in a mixture of tritiated thymidine, a precursor of *DNA*, hylauronidase, and Ringer's solution or Eagles medium[20]. The normal liver exhibits one to four labeled nuclei per 10,000 liver cells, a significant increase in *DNA* synthesis is usually noted in specimens from patients with active liver cell damage. Many patients with active liver disease and fluid and electrolyte imbalance fail to show the expected increase in *DNA* synthesis. Delineation of the mechanism responsible for impaired *DNA* synthesis is desirable, since ultimate control of fluid and electrolyte imbalance requires restoration of normal hepatic regenerative capacity. Depletion of vitamin B_{12}, folic acid, B_6 or protein has been found to interfere with *in vitro* hepatic *DNA* synthesis[21]. Hypokalemia and magnesium deficiency may also represent key defects which interfere with the reparative process. Correction of such defects may both restore electrolyte balance and initiate recovery from liver injury.

SUMMARY

Alterations in body water, serum electrolyte concentrations, acid base balance, and plasma volume are characteristic of liver failure. Early recognition of these abnormalities and provision of judicious supportive therapy are desirable for maintenance of body homeostasis. Improvement in hepatic functional status is of primary importance in restoration of normal water and electrolyte metabolism. This objective may be achieved with greater facility by use of laboratory methods to recognize electrolyte imbalance, serum pH changes and plasma volume alterations. Such investigations should be coupled with technics which evaluate hepatic reserve and detect abnormalities in the reparative potential of the liver.

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*Manufactured by the Waters Corporation.

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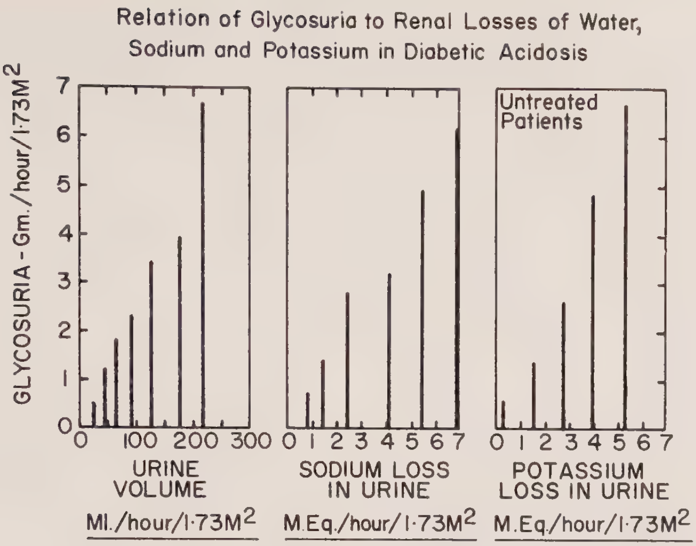
Electrolyte Metabolism in Diabetes Mellitus

JAMES B. FIELD, M.D.

The two situations in diabetic patients which are associated with disturbances in fluid and electrolyte metabolism are uncontrolled glycosuria and acidosis due to ketosis. Glucose in the urine provides an effective osmotic diuretic, as it has been clearly shown that the total urine flow is directly proportional to the total osmotic load presented to the kidneys[1, 2]. Brodsky *et al.* demonstrated that the diabetic was not dependent on any specific effect of glucose in terms of the osmotic diuresis and that the diabetic subject behaved the same as the non-diabetic in terms of renal handling of solute load of glucose, mannitol or urea[1]. As a consequence of the glycosuria, sodium losses as high as 1.5 mEq. per minute were observed in some diabetic patients. It is obvious that if this rate of sodium loss were to continue for any appreciable length of time, severe sodium depletion would soon result. Despite this marked loss of sodium, there was only a slightly increased loss of potassium as a result of the increased solute load. These studies were done in diabetic patients who were in good metabolic control except for the acute production of a glucose-induced osmotic diuresis. Therefore the authors' conclusions, that neither increased urinary flow nor hyperosmolarity of the plasma caused appreciable depletion of the intracellular electrolytes, must be restricted to the experimental situation which they were studying and not to the clinical condition associated with uncontrolled diabetes and acidosis.

Indeed, the studies of Nabarro and his colleagues on patients in diabetic acidosis demonstrated the direct relationship between the losses of sodium and potassium and the increased diuresis secondary to glycosuria[2]. There was also an excellent correlation between the urine volume and the total amount of glucose excreted (Figure 1). During diabetic acidosis, the osmotic diuresis is accentuated by the ketonuria with its attendant loss of electrolytes. As a consequence of this increased osmotic diuresis, the ability of the distal collecting tubules of the kidney to reabsorb water is reduced, and the maximal urinary concentration may fall to about half of the normal or 700 milliosmoles per l.[3]. Butler has estimated that the urinary excretion of solutes during diabetic acidosis may be as high as 3,500 milliosmoles per day[4]. This solute load would require a urinary volume of at least five l. in the presence of a



maximum urinary concentration of 700 milliosmoles per l. If fluid intake is maintained, the diabetic may be capable of supporting such large urinary volumes without developing dehydration, but as the acidosis progresses, nausea and vomiting restricts fluid intake, and these large fluid losses are not replaced. The resultant dehydration and hypovolemia further embarrasses kidney function and accentuate the diminished concentrating ability, necessitating a greater urine output for the same solute load.

The exact magnitude of the fluid and electrolyte losses observed during diabetic acidosis have been estimated by various investigators[2, 4, 5, 6, 7]. The pioneering studies of Atchley and colleagues are particularly noteworthy[5] and have been repeatedly verified by more recent investigators[2, 4, 6, 7]. A summary of the findings of these various investigators is presented in Table I. The remarkable similarity of the results obtained under somewhat different conditions over a period of over twenty years is obvious and reassuring. The studies of Atchley *et al.* conclusively indicated that all of the fluid and electrolyte losses were not due to acidosis *per se*[5]. During the first twenty-four hours of insulin withdrawal, their patient lost 0.8 kg. in weight and had an increase in urine output of 1,000 ml. Sodium excretion increased 87.1 mEq.

TABLE I ESTIMATED FLUID AND ELECTROLYTE LOSSES IN DIABETIC ACIDOSIS

AUTHOR	NO. OF PATIENTS	$Na+$ (mEq/kg)	$Cl-$ (mEq/kg)	HCO_3- (mEq/kg)	$K+$ (mEq/kg)	$Mg++$ (mEq/kg)	P (mM/kg)	H_2O (L/kg)
Atchley *et al.*	2	5.9	2.5	. .	4.9	. .	. .	0.089
Butler *et al.*	1	5.1	4.0	. .	5.6	0.8	1.3	. .
Danowski *et al.*	8	10.4	9.5	. .	6.0	. .	. .	. .
Nabarro *et al.*	7	7.2	5.1	. .	5.0	0.56	0.5	0.087
Martin *et al.*	8	7.0	4.0	4.7	2.7	0.16	1.0	0.082

and potassium excretion, 80.4 mEq. Calcium excretion increased 10.5 mEq. and chloride excretion, 66.8 mEq. There was also a negative nitrogen balance of 6 grams. All of these losses were observed before there was any significant change in the concentration of plasma bicarbonate, indicating that the losses were a direct consequence of the glycosuria and not acidosis. Furthermore, one of their patients had very similar fluid and electrolyte losses, even though he did not develop acidosis following the withdrawal of insulin therapy. In addition, a third patient, who did not develop significant glycosuria upon the withdrawal of insulin for four days, had no significant changes in water and electrolyte metabolism. The patient who did develop acidosis during the four-day period of insulin withdrawal lost approximately one kg. per day over a period of three days. On the second day of insulin withdrawal, sodium and potassium losses in the urine decreased slightly as compared to the first day, but then increased markedly to reach a peak on the fourth day.

Although there were increasing losses of chloride, phosphorus and sulfate in the urine during this four-day period, the total loss was not equivalent to the total loss of base. Most of the differences could be explained by the excretion of ketones. Nabarro *et al.* observed that the extracellular fluid and electrolyte losses were similar whether the acidosis developed slowly or rapidly[2]. However there were differences in the development of intracellular fluid and electrolyte losses. The more slowly the acidosis developed, the more profound were the aberrations in intracellular fluid and electrolytes. Sodium and chloride losses during the development of diabetic acidosis tend to be proportional to their concentration in extracellular fluid, being equivalent to about three l. of extracellular fluid (Table I). Martin reported that 67 per cent of a series of patients admitted in diabetic acidosis had low concentrations of serum sodium. Twenty-six per cent had normal concentrations of serum sodium; seven per cent had concentrations of sodium above normal[6]. Thirty-three per cent of these patients had decreased concentrations of chloride on admission. 45 per cent had normal values; 22 per cent had hyperchloremia.

It might be pertinent to mention a situation which is occasionally observed in patients with diabetic acidosis, which may influence the concentration of serum electrolytes. Albrink *et al.* emphasized the apparent lowering of the concentration of serum sodium in hyperlipemic serum and named this phenomenon hyperlipemic hyponatremia[8]. The elevated lipids displace water, and the concentration of sodium may be low if reported on the basis of 100 ml. of serum. However, if it is corrected for the displacement of serum water, the serum sodium will usually be found to be normal. They cite a patient whose sodium was reported to be 102 mEq. per l. of serum, but when corrected for displacement of serum water, was found to be 142 mEq. per l. This apparent decrease in the concentration of serum sodium due to hyperlipemia is also true for the other electrolytes and solutes in serum.

Before discussing the disturbances in intracellular fluid and electrolytes, the changes in the serum bicarbonate during the development of diabetic acidosis should be considered. As a consequence of the impaired carbohydrate metabolism in uncontrolled diabetes, there is an accumulation of ketone bodies and an increase in hydrogen ion concentration[9]. This accelerates the conversion of bicarbonate to carbonic acid, which spontaneously forms carbon dioxide and water. Respiration is stimulated in an attempt to remove this excess carbon dioxide, as well as to re-establish the normal ratio between bicarbonate and carbon dioxide in order to maintain a normal pH. The excess loss of base in the urine further contributes to the acidosis[5]. The kidney can compensate for the increased hydrogen ion concentration, either by exchanging hydrogen ion for sodium or by increasing ammonia production. However, in the presence of dehydration with decreased glomerular filtration, these functions may be impaired[3].

The studies of Atchley *et al.* conclusively indicated that the intracellular compartment also contributed to the deranged fluid and electrolyte metabolism in diabetic acidosis[5]. There are several factors which are of importance in the loss of fluid and electrolytes from the intracellular fluid. Initially, as a result of the hyperglycemia, there is a transfer of water from the intracellular to the extracellular space. Later, because of the depletion of extracellular fluid, there is an additional shift of water and with it electrolytes from the intracellular fluid in an attempt to maintain serum volume. Some indication of the magnitude of these changes is reflected by the observation by Sunderman and Dohan[10], that in pancreatectomized dogs allowed to go into coma, the serum volume per kg. of body weight was actually found to be consistently increased, even though the total volume of serum in the circulation was decreased[10]. Furthermore, secondary to the lack of insulin and deficiency of carbohydrate utilization, there is tissue breakdown and mobilization of protein to support augmented hepatic gluconeogenesis.

Concomitant with cellular breakdown, there is also loss of intracellular electrolytes and fluid. In the cells, for each gram of nitrogen, there are approximately 3 mEq. of potassium, 0.6 mEq. of magnesium and 1.5 millimoles of phosphorus[2]. However, the loss of potassium and water from the intracellular space is usually greater than can be explained on the basis of the negative nitrogen balance[4]. While the loss of magnesium is only slightly greater than the loss of nitrogen, the loss of phosphorus can be adequately explained on the basis of tissue breakdown and nitrogen loss[4]. Acidosis also influences the loss of electrolytes from the intracellular space[3]. The lowered intracellular pH causes a release of bound magnesium and potassium and a promotion of hydrolysis of organic phosphates. Mobilization of bone minerals, including bone sodium, is also favored. Since the bulk of the potassium in the body is intracellular, the losses of potassium during diabetic acidosis represent losses from this compartment (Table I). In a group of patients in diabetic acidosis the serum potassium was elevated in 39 per cent, normal in 43 per cent and decreased in 18 per cent[6]. Although there is a total body deficit of potassium, the increases in serum potassium can best be explained on the basis of hemoconcentration and the failure of the kidney to excrete potassium as fast as it is being lost from the intracellular space[11]. There is continued renal loss of potassium despite the presence of a body deficit[11].

In addition to the well-known and recognized consequences of hypokalemia on neuromuscular function, potassium deficiency may also have effects on carbohydrate metabolism[12, 13]. Potassium depletion in rats was associated with abnormalities of glucose tolerance tests and with decreased insulin responsiveness[12]. Sagild *et al.* reported that the experimental production of hypokalemia in humans was associated with deterioration of the glucose tolerance tests, but there was no impairment in the hypoglycemic response to a test dose of 0.1 unit of insulin per kg. A role for hypokalemia has also been suggested[14] and denied[15] in the deterioration of diabetes, which is seen in certain patients after the administration of thiazide diuretics.

Martin reported that 71 per cent of a group of patients admitted in diabetic acidosis had increased concentrations of serum phosphorus, 18 per cent had normal concentrations; 11 per cent had decreased concentrations[6]. Levitan indicated that glycosuria was associated with increased phosphaturia and that the bulk of the increased phosphate excretion was due to an increase in phosphate load presented to the kidneys with a minor component due to inhibition of reabsorption of phosphorus[16]. The role of phosphorus in the metabolism of glucose has also been studied, and the fall in serum phosphorus following the administration of glucose has been taken as an indication of peripheral utilization of glucose[17]. Forsham and Thorn noted that diabetic patients had a decreased fall in serum phosphorus after glucose as compared to the normal. Serum magnesium levels are also usually elevated in patients in diabetic acidosis; whereas, 25 per cent had normal concentrations, 7 per cent had decreased concentrations[6]. Serum calcium was normal in 68 per cent of these patients, increased in 4 per cent and decreased in 28 per cent.

Although the fluid and electrolyte changes are a direct consequence of the osmotic effect of glycosuria and ketosis in diabetes, the same may not be true of zinc metabolism. Constam *et al.* have reported increased zinc in the urine and higher fasting plasma values in diabetics without complications as compared to non-diabetics[18]. There was no correlation between the plasma zinc and the blood sugar levels or the type of treatment the patient was receiving. However, these findings could not be confirmed by Graig[19].

SUMMARY

In uncontrolled diabetes, there are major disturbances in fluid and electrolyte metabolism. These disturbances are primarily the result of the glucose-induced osmotic diuresis. Initially, there are losses of water, sodium and chloride from the extracellular fluid, but if the glycosuria continues, these losses are also reflected in changes in the intracellular compartment. There is a shift of fluid and electrolytes from the intracellular space to the extracellular one. In addition, there is breakdown of cells with liberation of potassium, magnesium and phosphate into the extracellular fluid. Although the majority of patients in diabetic acidosis have concentrations of serum potassium that are normal or high, they have a total body deficiency of potassium. Not only does uncontrolled diabetes lead to losses of fluids and electrolytes, but some of these losses may, in turn, impair carbohydrate metabolism and responsiveness to insulin.

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Disturbances of Electrolyte Metabolism in Bone Diseases and Parathyroid Diseases

LEO LUTWAK, Ph.D., M.D.

In addition to calcium and phosphorus present as the primary structural mineral (calcium hydroxyapatite), bone also contains other anions and cations, either functionally or fortuitously. The chemical composition of bone mineral reflects, qualitatively, the chemical composition of the extracellular fluid at the time of deposition of the bone crystal. Thus, chemical analysis of the skeleton yields an indication of the nutritional and pathophysiological status of the organism at the time of bone formation. Table I shows a composite analysis of human bone, indicating the multiplicity of ions which may be found. The metabolic activity of bone, as discussed in Chapter 22 results in both uptake of material from extracellular fluid in the process of bone deposition, and release of previously deposited substances in the course of bone resorption. Therefore, disease of the skeletal system can result in reflected disturbances of various anions and cations.

TABLE I BONE COMPOSITION

ELEMENT	PER CENT OF DRY BONE
Ca	25.6 — 27.2
Mg	0.39 — 0.44
Na	0.18 — 0.73
K	0.05 — 0.3
Fe	0.011 — 0.017
Cu	0.0002 — 0.0048
Pb	0.001 — 0.01
Mn	0.00005 — 0.0022
Sn	0.00005
Al	0.00024 — 0.0005
Sr	0.00001 — 0.0002
Bo	0.0016 — 0.014
P	12.3 — 12.5
CO_3	3.4 — 4.0
Cl	0.07 — 0.19
F	0.061 — 0.31
S	0.14

A discussion of the contribution of skeleton to the maintenance of electrolyte homeostasis should be preceded by consideration of the chemical reactivity of the skeletal mineral as affected by aging and disease. The mineral phase of bone has been represented diagramatically in Figure 1, following the conceptualization of Neuman and Neuman[8]. The outer-most surface of the crystal consists of a hydration shell, similar in composition to, and in complete equilibrium with, the extracellular fluid of the organism. Next to this outer hydration shell is the surface of bone crystal, which can still exchange readily with the hydration shell immediately surrounding it. Deeper within the crystal structure are older, previously de-

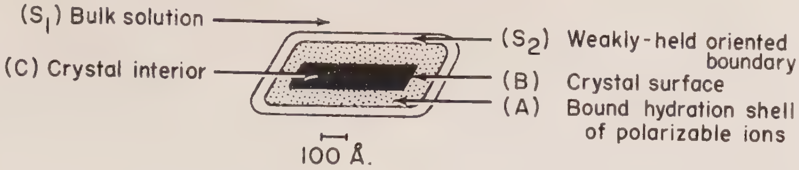
(S₁) Bulk solution
(C) Crystal interior
(S₂) Weakly-held oriented boundary
(B) Crystal surface
(A) Bound hydration shell of polarizable ions
100 Å.

Fig. 1. Reprinted from Neuman and Neuman[9], by permission of the publishers, University of Chicago Press, 1958.

posited crystals which are less hydrated and less readily exchangeable with the immediately adjacent area. Thus, effectively, the chemistry of bone crystal becomes a problem of surface chemistry of the crystals.

From x-ray crystallography we have learned that hydroxyapatite crystals are hexagonal plates of very small size, of the order of 200 x 30 — 70 Å. Each of these crystals, in turn, is composed of about 2500 unit "cells" of calcium hydroxyapatite with a theoretical formula of $Ca_{10}(PO_4)_6(OH)_2$. The bonding energies at the surface of a newly deposited crystal are sufficiently low that other cations may substitute for the calcium, and other anions may replace hydroxyl groups and phosphates in the course of crystal formation. In addition, Posner has described a pseudo-apatite or deficient apatite with "defects" in the crystal lattice accounting for non-stoichiometric proportions of calcium and phosphate[9]. These defects, which occur in the course of crystallization, may be subsequently filled by other cations. In the course of mineralization of the bone, hydroxyapatite may contain as substituents for the calcium, other cations such as barium, strontium, magnesium, radium and other alkaline earths; in place of phosphate, carbonate, citrate, uranate; in place of hydroxyl, fluoride, thus yielding the heterogenous mineral usually isolated from bone. In addition, sodium may enter the bone crystal, both in solution in the solid phase and by exchange with calcium. There are four main types of ion interactions with hydroxyapitite crystals[8]:

(a) Ions which diffuse into the hydration shell but do not concentrate there. A steady state is attained rapidly. The system is readily reversible, and the concentration of the ion in the hydrated solid is directly proportional to its concentration in the surrounding solution.

(b) Ions which enter the hydration shell and participate in neutralization of surface charge asymmetry. A steady state may be attained in a few hours. The system is readily reversible, and the concentration of the ion of the hydrated solid is a function of its concentration in the solution relative to the concentration of the ion being displaced, such as calcium, phosphate or hydroxyl.

(c) Ions which enter the hydration shell and replace ions in the crystal surfaces. Again the steady state may be attained within a few hours. The system is readily reversible, and the concentration of the ion in the hydrated solid is a function of its concentration in the solution relative to the concentration of the ion being displaced.

(d) Ions which can displace lattice ions both in the surface and in the

> crystal interior. A steady state cannot be demonstrated over periods of months. The system appears to be irreversible, and the concentration of the ion in the solid phase is a function both of its concentration relative to the concentration of the ion being displaced and of the equilibration.

From these considerations, it may be predicted that only a portion of the total ion content of the skeleton can play a role in patho-physiology of electrolyte disturbances: that portion which is available for ready, rapid exchange processes in the hydration shell and in the surface of the crystals.

CALCIUM

Approximately 8 to 20 per cent of the skeletal calcium is available for exchange[4, 10]. This exchangeable fraction is the amount of calcium present in the hydration shell, the crystalline surface, and, perhaps, some from deeper layers.

MAGNESIUM

About 5 to 40 per cent of the skeletal magnesium is exchangeable[2, 5], and, therefore, available for physiologic regulation processes. This, too, is present in the hydration shell and the crystalline surface, decreasing as the individual (and his skeletal mineral) ages.

SODIUM

About 25 per cent of bone sodium is exchangeable with extracellular fluid sodium[1, 3, 7], again, probably representing that within the hydration shell and on the surface. The non-exchangeable bone sodium is that which has been deposited in the course of the formation of older bone crystal.

STRONTIUM

This cation enters into the skeletal metabolism in a pattern similar to that for calcium and, thus, approximately 10 per cent is exchangeable.

POTASSIUM

This ion diffuses into the hydration shell but does not concentrate there, nor undergo exchange with the crystal surface[8]. Therefore, its concentration in bone is quite low, and most of that which is present is readily exchangeable.

OTHER CATIONS

These include primarily adventitiously ingested materials such as lead and radium which behave similarly to calcium. In addition, there are trace concentrations of zinc, manganese, cobalt, iron etc., the behavior of which is not too well understood at present.

PHOSPHATE

The availability of skeletal phosphate is similar to that of calcium. As mineral is resorbed, both calcium and phosphate are equally released into the extracellular fluid.

CARBONATE

One of the most important displacements is that of phosphate ions by carbonate ions. The carbonate may be present in the mineral either as a separate phase of calcium carbonate or as a replacement for phosphate in the hydroxyapatite structure. The surface carbonate is available for physiologic demand in the process of exchange of hydration shell and crystal surfaces.

CITRATE

Approximately 90 per cent of the citrate of the body is in the skeleton where it may account for as much as 1 per cent of the fresh weight of bone. Because of the size of the citrate molecule, it clearly cannot exist within the apatite structure and must be present adsorbed to crystalline surfaces. Only a portion of the total bone citrate is readily exchangeable and available and, therefore, the remainder is adsorbed to deeper layers of bone mineral.

FLUORIDE

Replacement of the hydroxyl group of calcium hydroxyapatite results in the formation of a more stable crystal, calcium fluoroapatite. The majority of the fluoride present in bone is in this form, and that which is located deeper within the crystal is not available for exchange and utilization elsewhere in the body. The fluoride present adsorbed to crystalline surfaces dissolved in the hydration layer and within the superficial crystals which are under going resorption is available.

CHLORIDE

Like potassium the chloride ion diffuses into the hydration shell but does not concentrate there. Thus its concentration in bone is quite low and primarily exchangeable.

With these considerations in mind, the effects of skeletal disease on electrolyte metabolism may be considered. Since bone exhibits stereotyped responses to given stimuli, skeletal pathology will be considered under three general categories:

(a) Disorders in which the amounts of bone have been diminished but in which bone turnover is relatively normal.

(b) Disorders with rapid bone turnover.

(c) Disorders with decreased bone turnover.

1. *Normal Kinetics.* This group of diseases include the various forms of osteoporosis in which bone resorption may be relatively greater than bone formation, but the process is so gradual that many years are necessary for pathology to appear. In this group of disorders, no abnormalities of systemic electrolyte metabolism are seen. The total skeletal stores of calcium and phosphorus are diminished, as are the stores of other ions. Undoubtedly the total skeletal reserve of magnesium and sodium is diminished, but this is not reflected in the homeostatic concentrations in extracellular fluid. Predictably, under situations of extreme demand, such as may occur in an individual parenterally fed for long periods of time, systemic deficiencies of magnesium may occur.

2. *Disorders of Increased Turnover.* This group includes hyperparathyroidism, Paget's disease of the bone, and possibly hyperthyroidism. Under these circumstances, the skeleton contains a greater proportion of exchangeable material, because more and more of the mineral is newly deposited and does not have an opportunity to age into nonexchangeable mineral. Since phosphate is being lost from the body at an accelerated rate in hyperparathyroidism, bone is resorbed to a greater extent than deposition can occur, and excess calcium is present in the serum. Total body reserves of sodium are increased; more sodium becomes available for excretion, and hypernatriuria is frequently seen. In acute hyperparathyroidism, the serum concentrations of magnesium may be markedly elevated because of the rapid

dissolution of bone mineral. Conversely after long-standing hyperparathyroidism has been treated by surgery, the skeleton begins to remineralize in the absence of the continuing action of the parathyroid hormone. Under these circumstances, serum concentrations of various cations such as magnesium may fall precipitously and induce hypomagnesemic irritability[6]. In Paget's disease similarly, a great proportion of the skeleton becomes exchangeable because of rapid breakdown and resynthesis. When the stimulus for new bone formation is removed, as may occur when a patient with Paget's disease is put to complete bed rest, bone resorption will continue. Serum calcium and magnesium become markedly elevated, sodium appears in the extracellular fluid to high concentrations, and cardiac failure may result.

3. *Disorders with Low Turnover.* These include, primarily, hypoparathyroidism and occasionally myxedema. Because of the low rate of turnover, the greater proportion of the skeletal mineral is non-exchangeable and, therefore, not available for body demands. Hypomagnesemia may occur as well as the well-recognized hypocalcemia.

SUMMARY

Since the mineralization of the skeleton occurs through the crystallization of calcium hydroxyapatite from a heterogeneous solution of extracellular fluid containing many other ions other than calcium and phosphate, and since the crystal structure of this mineral is based upon a loose lattice, the resulting substance is heterionic, containing amounts of other electrolytes such as sodium, magnesium, carbonate, chloride, fluoride, etc., reflecting the physical-chemical nature of the crystal and the chemical composition of the environment at time of formation. In the process of crystal aging, 70 to 90 per cent of the mineral becomes unavailable for exchange with extracellular fluid. That which remains can be called upon to replenish losses from the extracellular space. In pathophysiology of the skeleton the total amount of mineral may be diminished, or the availability for exchange may be either increased or decreased, and thus result in generalized disturbances of electrolyte metabolism.

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Electrolytes in the Pathogenesis of the Periodic Paralyses

G. MILTON SHY M.D.

The Periodic Paralysis

Severe muscle weakness is an episodic feature of many disorders such as renal shutdown, diabetic coma, primary aldosteronism, Addison's and Cushing's diseases. All of these disorders may also have at the time of paralysis, severe cationic disturbances. The potassium ion is the cation most consistently involved.

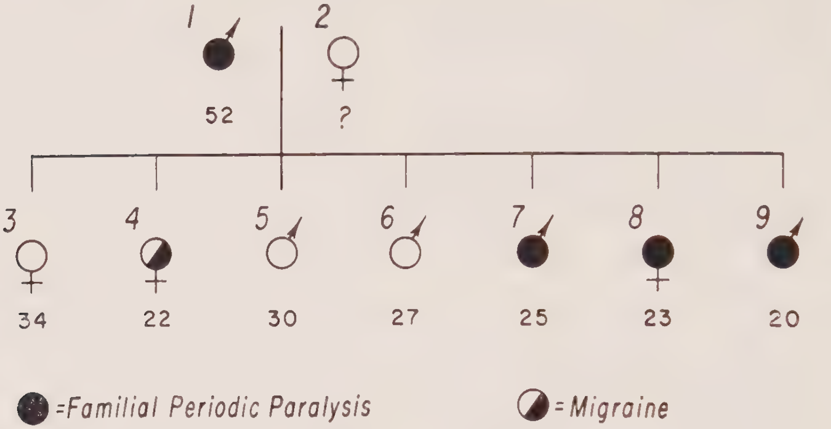
There are two disorders characterized by spontaneous flaccid paresis in which the causative agent is still unknown. Both are transmitted as autosomal single dose traits (Fig. 1). In one, familial periodic paralysis, there may be a hypokalemia with the attack and in the other, paramyotonia congenita, there may be a hyperkalemia. Each of these two disorders is also closely dependent upon carbohydrate metabolism. Recent intracellular recordings[14, 22], electron-microscopy[22], endocrinologic studies[3], cytochemistry, pathology, and isotopic studies have given new insight into these disorders.

FAMILIAL PERIODIC PARALYSIS

Familial periodic paralysis is a disorder characterized by episodes varying from quadraparesis to weakness of part of an extremity only. Associated with the paresis is a decreased urinary output associated with occasional acetonuria and cyclindruria. The swelling of the involved extremities has been noted periodically for a number of years in the literature. Attacks may be precipitated by rest and ingestion of carbohydrates. The majority of attacks occur at night and the usual finding is that of paralysis on waking in the morning. If the attacks are severe, then marked cardiac arrhythmias may occur. The bulbar musculature is spared except in the most severe attack. The frequency of attacks varies with the individual and attacks may occur nightly or be as infrequent as one per year. Some patients may have but two or three attacks in their lifetime. If the attacks are frequent, it is not uncommon to find permanent residual weakness and wasting of proximal musculature later in the patient's lifetime. In the majority of attacks there is an associated hypokalemia and hypophosphatemia. Leukocytosis and eosinophilia have been reported. There is a greater frequency of migraine in both the afflicted individuals and in the non-afflicted family members than may be accounted for by chance. During attacks of paresis, the muscles affected do not respond to either galvanic or faradic stimulation[25].

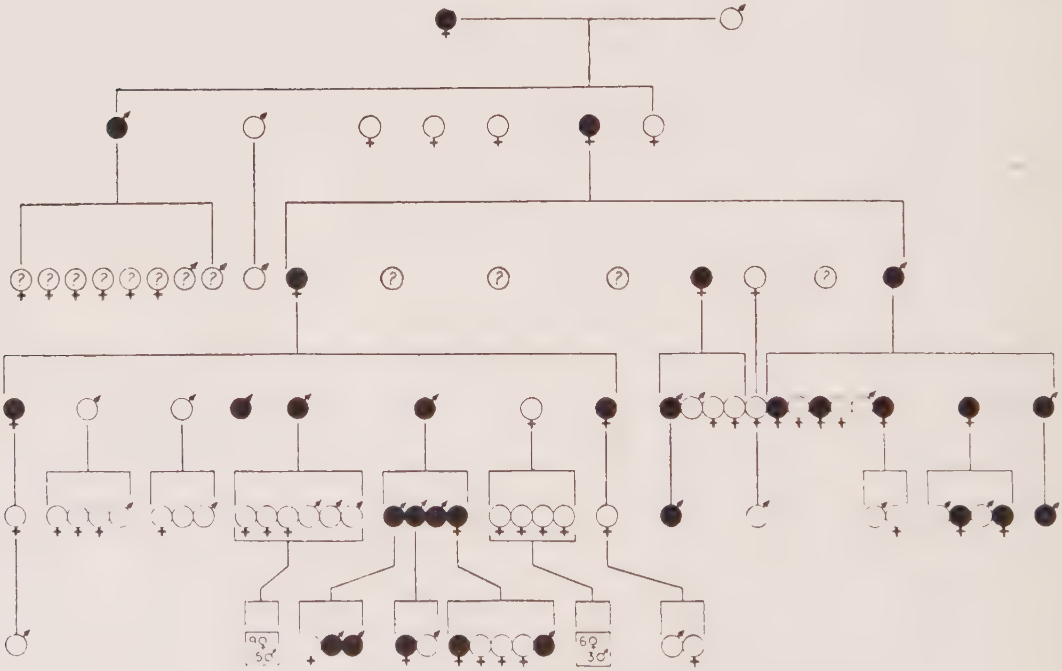
Pudenz *et al.*[16] and Gammon[5] have demonstrated a prophylactic effect of

Fig. 1. Pedigrees of typical autosomal single dose patterns of inheritance in the periodic paralyses.



Pedigree of "Q" family.

Shy et al (1961)



Pedigree of X. family. The black represents the members with paramyotonia. Circles with the question mark represent those members for whom paramyotonia is in question.

Drager et al (1958)

potassium chloride. Whether the reversal of paresis upon administration of KCl is valid or whether the normal termination of the attack is coincident with the administration of KCl is still open to question. There appears no doubt from arterial-venous differences in isolated extremities that K does enter the muscle cell during the attack. However, the extracellular K^+ concentration

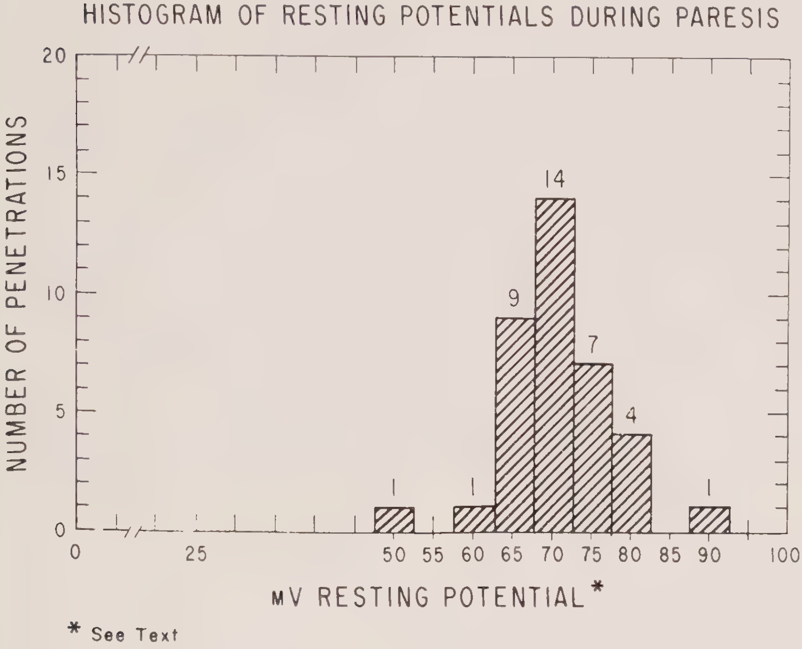
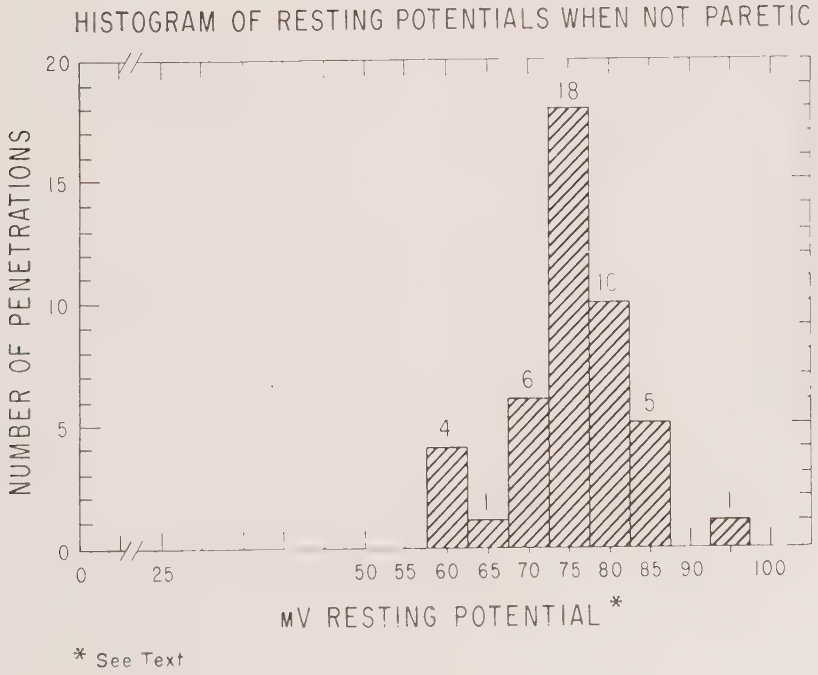


Fig. 2. Intracellular resting potentials during and after paresis. Shy et al. (1961)



is only 5 mEq. per liter, whereas the intrafiber K^+ concentration is of the order of 115 mEq. per liter.[9] Assuming a maximum extracellular water compartment of 20 per cent (12 per cent Horvath *et al.*)[9], if the serum K fell from 5 to 1 mEq. per liter, less than 1 mEq. would enter every kgm. of muscle. This would shift the intracellular K concentration from 150-151 mEq. per liter. Using the Hodgkin-Huxley[8] equations, the resting potential would then be $Er = 58 \log \frac{1}{151} = -151$ mV whereas if the same serum K were 5 mEq. then the $Er = -92$ mV since $Er = 58 \log \frac{5}{151}$ (mV). This amount should not, however, make the cell refractory to firing.

However, when measured with intracellular pipettes during and after paresis no significant mV. shift is detected and the cell is indeed refractory to the passage of propagated potential on both direct and indirect stimulation (Fig. 2). Spectrophotometric determination in which standards are set for 150 mEq. will not reflect a 1 mEq. change. When samples are taken from such a patient during or between attacks and corrected back to the dry weight or N.C.N., then it is true that no increase in intrafiber K^+ may be seen (Fig. 3). This does not mean that the one mEq. of potassium did not enter the muscle cell as clearly shown by the A-V difference within the limbs. This does mean, however, that another reason for the paralysis other than excessive polarization of the muscle cell must be sought since by actual recording there is no significant change in the resting potential.

TABLE NO. I.

	CONTROL	PARALYTIC	kgm
1000:	257 : x : 286 ⁻ 1,111		
Dry wt	286	257	
Total H_2O	714	743	
K/kilo muscle	79	68.5	76
Na/kilo muscle	48	41.0	45.5
Cl/kilo muscle	35.5	35.1	39.0
K/liter serum	5.25	2-4.1	
Na/liter serum	139	140	
Cl/liter serum	113	104	
μc/mEq K muscle	.0577	.0559	
NCN	22 gms.	20.5 gms	
K_E exchangeable (urine)	1695	1403.9	
K_E exchangeable (urine)	2864	2624	
K_E exchangeable (muscle)	2755	3234	
24 hr. period			
Urine output	3267	1929	
K mEq	437.2	105.4	
Na mEq	237.3	138.2	
K μc	% Fall 24 hr. comp. = 54%		
K mEq	% Fall 24 hr. comp. = 41%		
Blood K	53.5	60.5	
Intake K	5500 mgm + 11000 mgm	5500 mgm	
Na	3.0G =	3.0G	
Intake in mEq K	420 mEq	140 mEq	
Na	125 mEq	125 mEq	

Fig. 3. (Table I, Shy et al., 1961) Major cation concentrations and isotopic kinetics in familial periodic paralysis.

Recent chemical, histochemical, and cytochemical studies performed upon patients during and between attacks of periodic paralysis have shown that the muscle fiber water increases by 11 per cent during an attack[22] (Fig. 3). Such fluid appeared on microscopic sections to be loculated within the involved fibers (Fig. 4). Such loculation would indicate that the fluid was contained within membranous elements. Periodic acid-Schiff material was also found in such fluid accumulations. This, in turn, could be erased by the application of diastase.

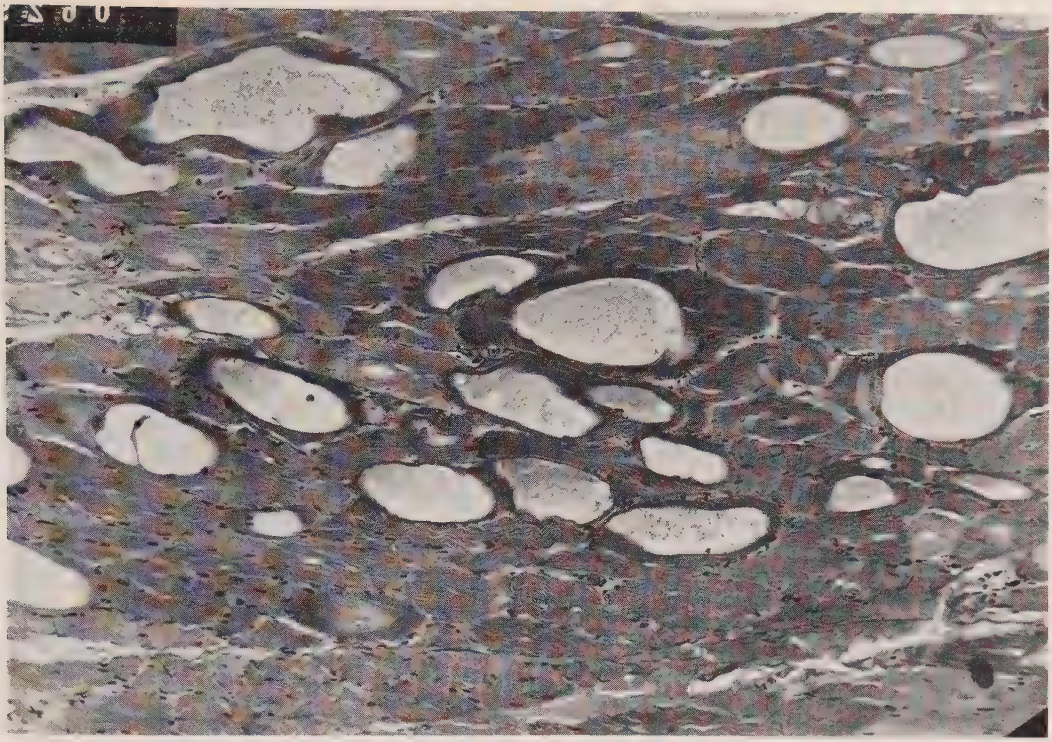


Fig. 4. A typical microscopic field of muscle fibers during a paralytic attack. Note intracellular fluid loculations. X480 — Gomori trichrome.

To clarify the situation further, ultrastructure studies were carried out at various phases of paralysis and the fluid was seen to be contained within the endoplasmic reticulum (Fig. 5). Mitochondrial elements were also seen to undergo profound changes during the paresis (Fig. 6). All of these changes were reversible during remission although small loculations of fluid were always seen[22]. Such loculations have been reported by almost all observers. More recently, however, a case of hypokalemic paresis was noted in which such loculations were not noted. This may represent the random biopsy but, if not, would cast considerable doubt on the validity of distention of the endoplasmic reticulum as a major contributing factor to the paresis[18].

Conn[1] recently presented the hypothesis that an abnormality of aldosterone excretion is a major contributing factor in this illness. Rowley and Klinman[17] pointed out, however, that if this were true, then the subject would be in negative potassium balance and secondly, that attacks should be less frequent at night, when aldosterone secretion is the least. The converse is true however. Concomitant with the paresis, the patient becomes oliguric or anuric and hence goes into a positive potassium balance. The attacks also, as noted above, are more frequent in the night. Rowley and Kliman[17], however, measured the aldosterone excretion by the double isotope derivative technique and found no increase of aldosterone either before or during attacks. The plasma volume is one of the main contributing factors for excitation of aldosterone release.

Recent studies[22] have demonstrated no significant shift in either plasma or blood volume using isotopic Cr^{51} and I^{131}. Finally, the giving of pitressin to change the blood volume does not precipitate an attack unless large amounts of carbohydrates are given as well. Intravenous infusions of 0.2 mg. of aldosterone increased the excretion of aldosterone to 21 mg. per day and 0.3 mg. increased the excretion to 53 mg. per day, but neither precipitated attacks of paresis. When 9 cc. fluorohydrocortisone was given aldosterone excretion fell to 4 mg. per day. SC 8109 increased the excretion to 23-37 mg. per day. Neither precipitated an attack. Thus, no evidence could be found by these investigators to implicate aldosterone as an etiological agent in this disorder.

The influence of carbohydrates in the precipitation of attacks of familial periodic paralysis has long been known[23]. A heavy ingestion of carbohydrate, insulin, or adrenalin may precipitate an attack in a susceptible individual. It has also been long recognized that there is a delayed glucose rise in the glucose tolerance curve[23, 22].

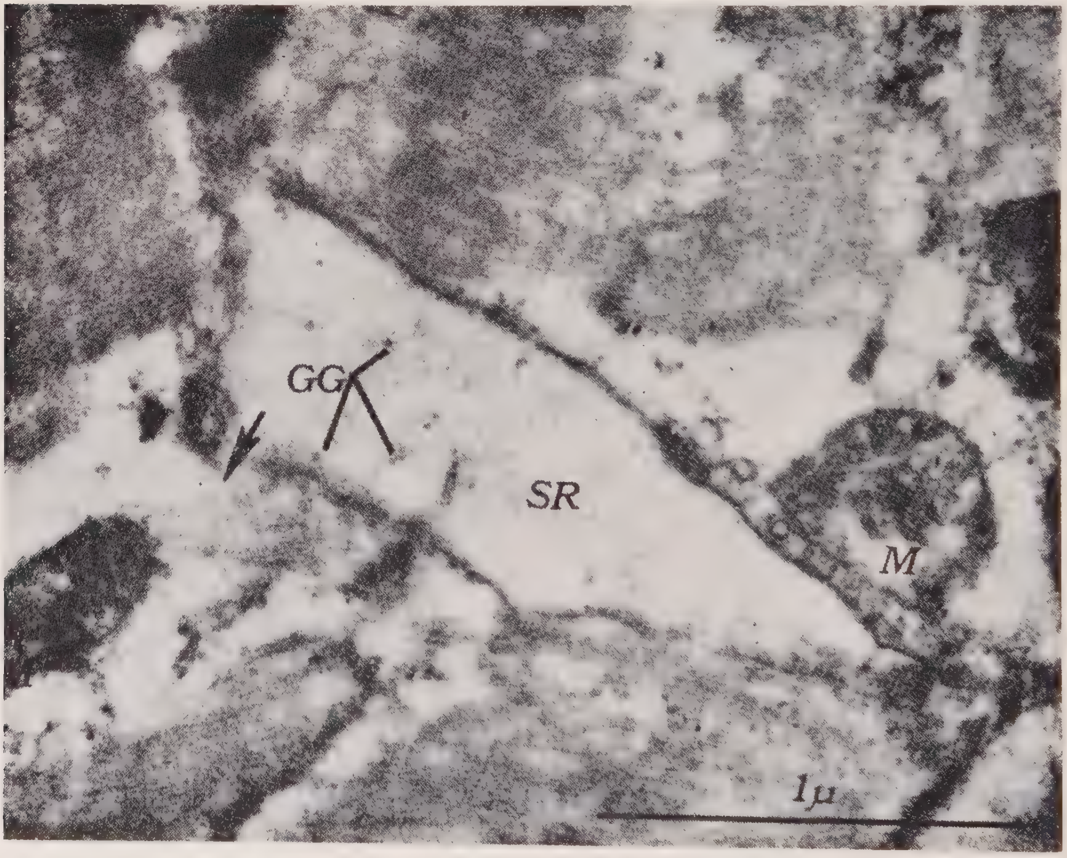


Fig. 5. Smaller loculations are seen to be in the endoplasmic reticulum. Note membrane X 18,000, osmium tetraoxide.

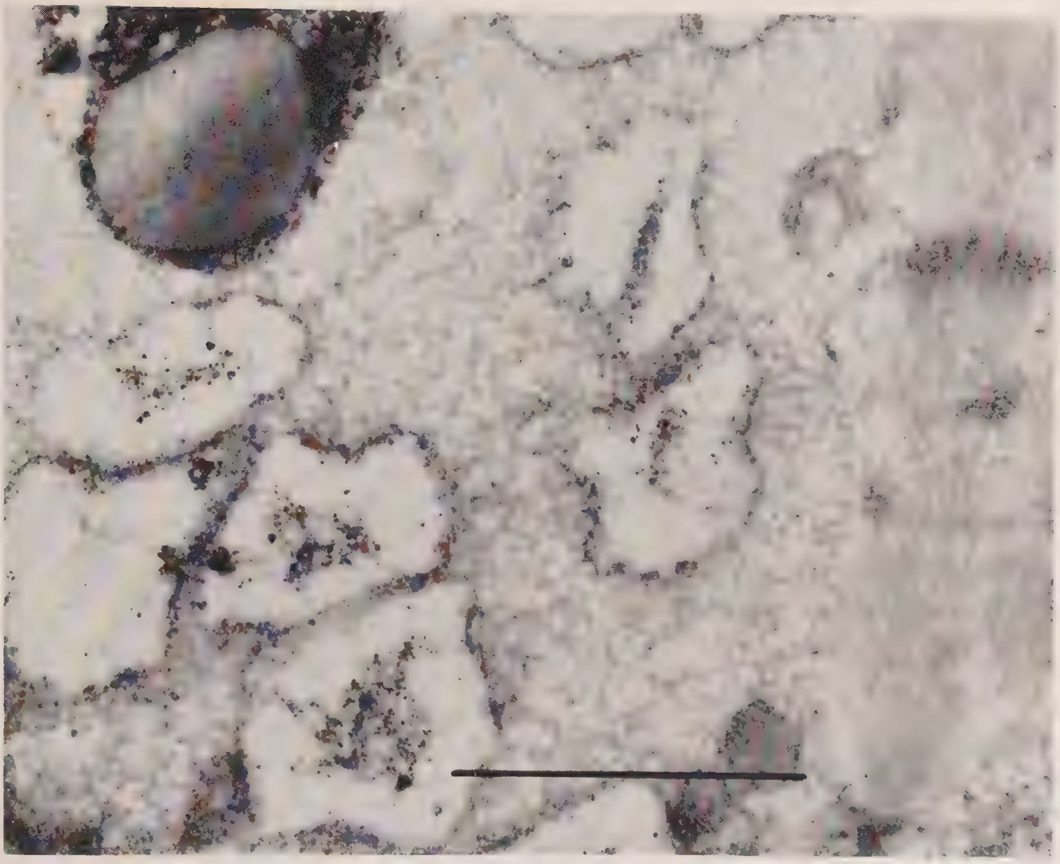


Fig. 6. Mitochondrial swelling and disappearance of cristae during attack of paralysis, X 36,000, osmium tetraoxide. Shy et al. (1961)

The entrance of glucose into the muscle cell is accompanied by a movement of potassium. Several recent studies have emphasized the effects of carbohydrate metabolism in this disorder. McArdle[12] indicated a possible partial block in the hexose phosphate stage of carbohydrate metabolism. This could cause an excessive accumulation of non-diffusable phosphorylated anions and an influx of potassium in the muscle. This would occur both during the breakdown and synthesis of glycogen. This concept could not, however, explain the protective effects of exercise which favors muscle glycogen breakdown and which during attacks will cause an abnormally high efflux, not influx, of potassium. More recent work indicates that glycogen synthesis and breakdown may occur along separate pathways to and from glucose-1-phosphate[19]. Thus, glucose-1-phosphate reacts with uridine triphosphate. Glycogen synthetase then transfers the glucose residue from UDPG to the amylose chain and pyrophosphate. Further polymerization of glycogen depends on the branching enzyme which connects the amylose chain by alpha-1,6-linkages, Such a path is reversed by debranching enzymes and phosphorylase. The reaction with phosphorylase to form glucose-1-phosphate depends upon 3′,5′-adeno-

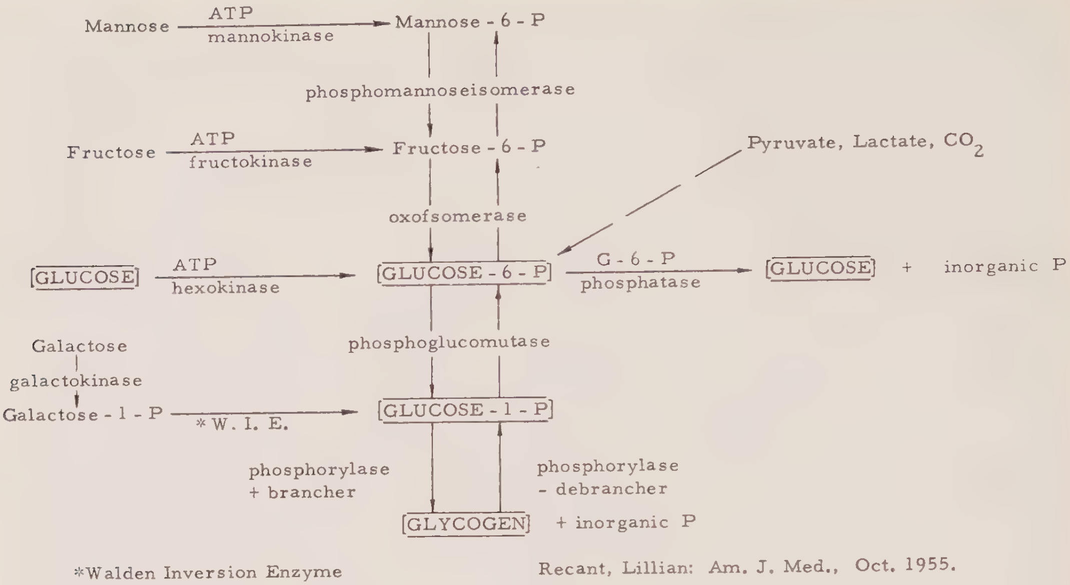


Fig. 7. The position of phosphorylase in glycogenolysis.

sine-monophosphate (Fig. 7). The formation 3′,5′-AMP is accelerated by epinephrine muscle and by epinephrine and glucagon in the liver. According to our present knowledge glucagon mobilizes glucose through liver glycogen and hence increases the peripheral utilization of glucose in proportion to the induced hyperglycemia. Glucagon has no effect at the muscle cell. Epinephrine, however, mobilizes liver glycogen as well as enhances glycogenolysis in the muscle. On the other hand, glucocorticoid hormones may be expected to increase hepatic and muscle glycogen storage and to augment the hepatic glucose output. Thus, a combination of these four elements may give some indication as to an abnormality of glucose metabolism in this particular disorder. Shy *et al.*[22] studied patients with this disorder on a constant diet of 2,000 calories consisting of 250 gms. of carbohydrate, 70 gm. of protein, and 80 gm. of fat, with the dietary sodium and potassium intake held constant. The initial study utilized the glucocorticoid triamcinolone (Fig. 8). Levels up to 30 mgms, per day were given for periods of two to five days. This glucocorticoid readily produced attacks of severe nature during administration. It is of interest that some of the severe attacks were produced when the serum potassium was between 4 and 6 mEq. per liter. Thus, by this study one could anticipate either increased hepatic or muscle glycogen storage and an augmentation of hepatic glucose output. To clarify this further, these investigators next utilized repeated dosages of glucagon and controlled this in normal patients without this disorder. The glucagon was administered in a dose of .003 mg. per kilo of body weight. Upon administration of glucagon to patients with familial periodic paralysis a rapid rise to 100 mg. per 100 ml. above the fasting blood sugar level was experienced. Upon repeated dosages of

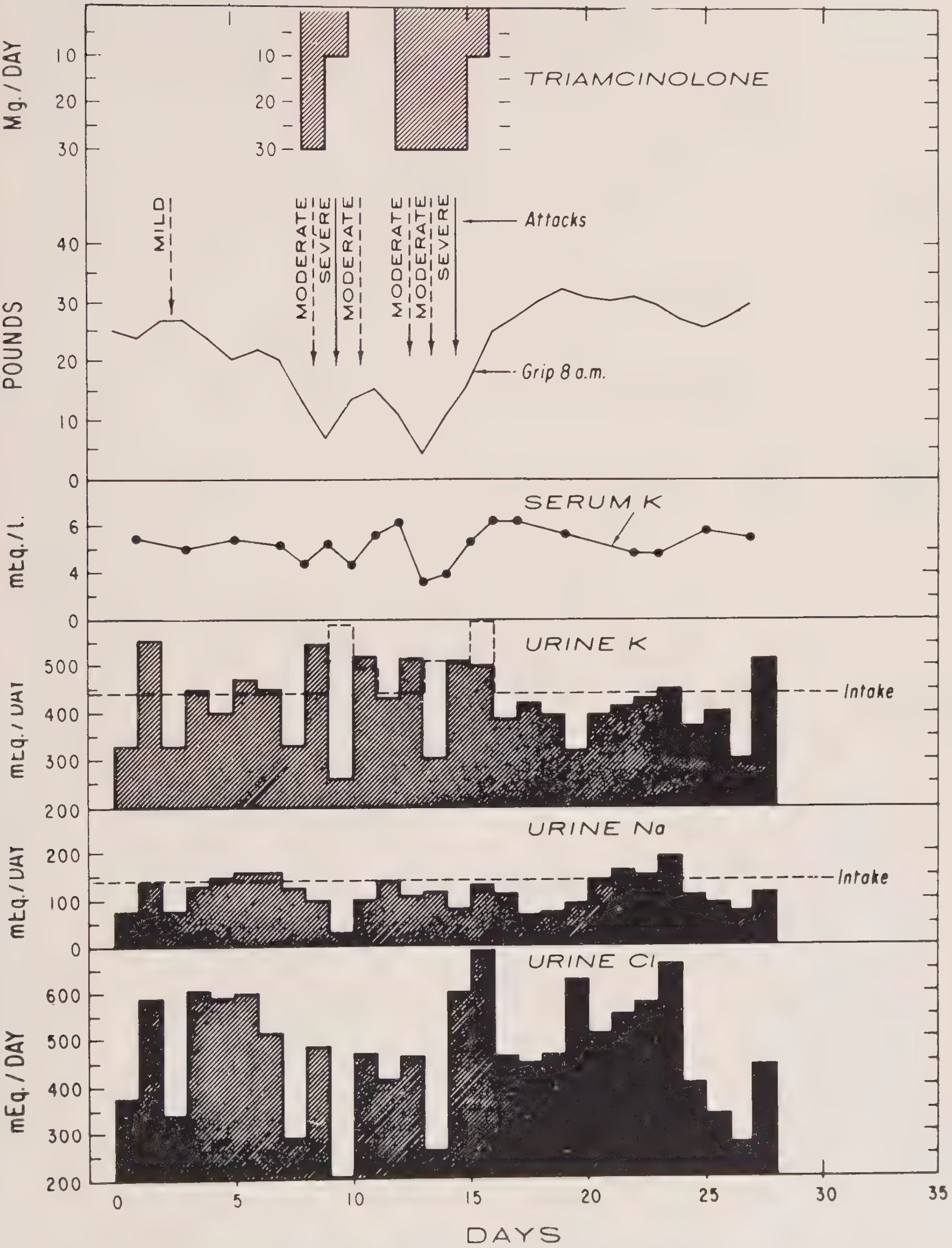


Fig. 8. The effect of a glucocorticoid on familial periodic paralysis. Shy et al. (1961)

glucagon, however, no secondary rise could be found nor was paresis evident. When both epinepherine and glucagon were given, an elevation of approximately 200 mg. of glucose above the fasting level was found and a beginning secondary rise was detected upon repeated dosages. Now early paresis was evident. When the patient was exercised and glucagon and epinepherine given, then the usual repeated rises were seen upon each injection of glucagon and profound paresis ensued. Again, the serum potassium was frequently within normal limits (Fig. 9). Since glucagon did not given secondary rise as it did in normal individuals, and finally, since secondary rises were seen when epinepherine was used, these investigators concluded that the glycogen depot of the liver was readily depleted in such individuals, and it was only when secondary metabolities were released from muscle that secondary rises in the blood sugar could be found upon repeated dosages of glucagon. This would indicate that there may be an excessive storage of glycogen within the muscle fiber which could be released by epinepherine but that there was a decreased storage of glycogen in the liver in such patients. This study further indicated that only when muscle glycogen was broken down did paresis ensue in such individuals. These findings combined with the localization of

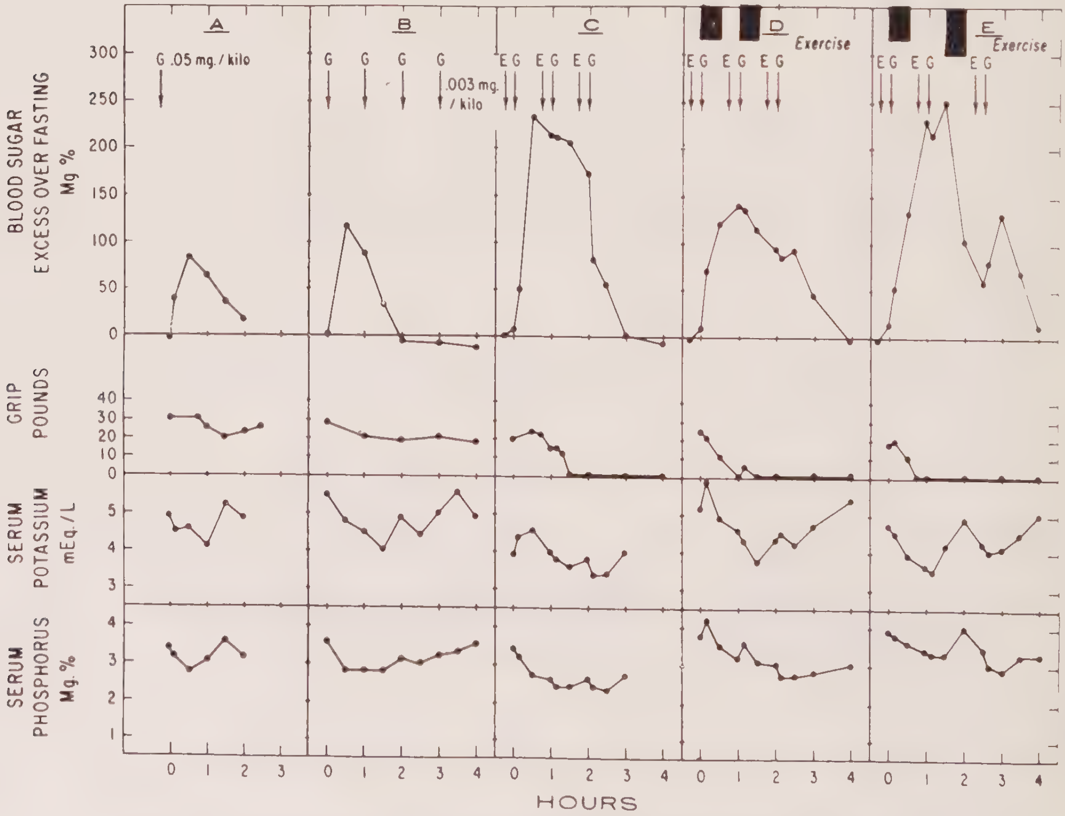


Fig. 9. The response of a patient with periodic paralysis to glucagon. Shy et al. (1961)

PAS-positive material within the vacuoles of the affected muscle during paresis indicates that there may be indeed an abnormality of the carbohydrate system in patients with familial periodic paralysis. This is further indicated by the delayed rise in glucose upon the glucose tolerance curve. This has been subsequently confirmed by Norris[14].

It would appear that there is an imbalance in glycogen storage between liver and muscle with a diminished storage of glycogen at the hepatic tissue and perhaps excessive storage within the muscle fiber during periods of attack. Since the muscle glycogen may readily be broken down and released by epinephrine and exercise, and since exercise itself is a prophylactic agent against the attack, it would indicate that the abnormality is on the anabolic side of glycogen metabolism. Since hepatic tissue itself seems not to have its normal content of glycogen for release it would indicate in such patients that such glycogen is transferred and stored in the muscle cell instead of the hepatic cell. Since the liver is not free then to release the glycogen for utilization by the muscle cell, glucosteroids then would accentuate the abnormality of the weakness as would epinepherine which works at both sites. The combination of glucagon and epinepherine would again allow a resynthesis of glycogen at the hepatic level but the continuing abnormality of storage at the cellular level in the muscle would once again precipitate attacks. This would indicate that the potassium is merely a reflection of the passage of carbohydrate through cell membranes and that paresis could occur with or without an abnormal potassium level reflected in the serum. The accumulation of abnormal metabolites within the muscle cell in turn would cause an ingress of water as well as potassium into the cell and this in turn, if localized in the endoplasmic reticulum, would increase the surface area to the extent that the total resistance of the membrane would not permit propagation of the action potentials[18]. Whether or not this thesis can explain all facets of this complex disease remains for further studies[20]. In particular, studies should be done in the enzymatic defects which may be present in the Embden-Meyerhof glycolytic chain.

Complete phosphorylation is dependent upon intactness of the mitochondria. As noted above, the mitochondria are affected during the acute periods of paralysis. It is now recognized that thyroxin has its major effect on the mitochondria of muscle cells and a recent muscle abnormality has been described by Luft *et al.*[11] in which hypermetabolism is associated with abnormal giant mitochondria within the muscle cell itself. It has been long recognized, particularly in Japan, that there is a form of sporadic periodic paralysis associated with thyrotoxicosis[7, 21]. To further elucidate this in the familial form of periodic paralysis *Engel*[3] accomplished a remarkably lucid study in which he segregated the effects of hypermetabolism, TSH, and thyroxin. The patient was first placed on tri-iodothyronine at levels of 100 mcg. for ten days. By measuring the radioactive iodine uptake, Engel was able to demonstrate that at this level there was a depression of thyrotropic hormone in the pituitary. At this point, he then increased the tri-iodothyronine to 1,000 mcg. per day and made the patient

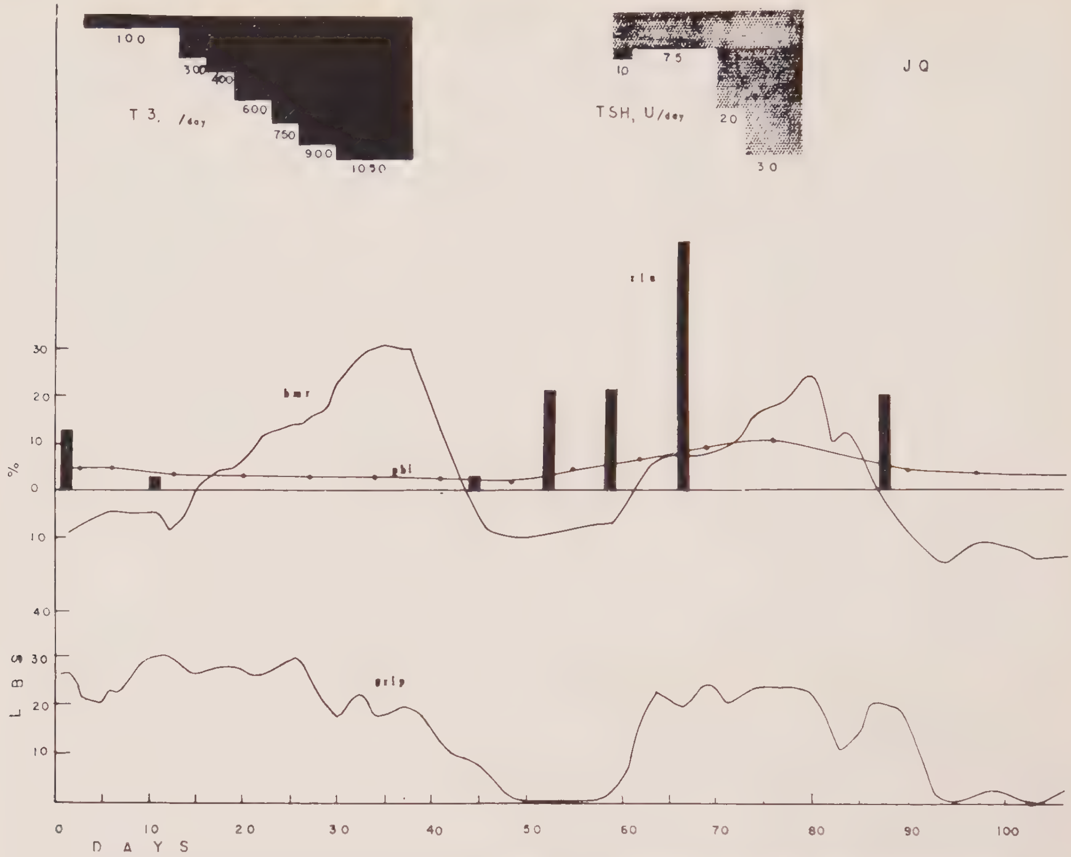


Fig. 10. The results of the interaction of thyroidial and thyrotropic factors and the metabolic rate in a patient with periodic paralysis (after Shy et al., 1961).

deliberately thyrotoxic. No weakness was noted. The medication was then suddenly stopped and with the subsequent fall in the basal metabolic rate the patient became remarkably weak. To ascertain if this weakness were due to the falling metabolic rate or to the subsequent rise of released TSH, Engel then deliberately administered exogenous TSH and once again made the patient thyrotoxic. Muscle power returned at this time. Thus, Engel was able to conclude that the weakness of familial periodic paralysis is a reflection of the metabolic rate of muscle and not of the thyroid gland or TSH (Fig. 10). Engel's studies have subsequently been confirmed by Norris[14].

Thus, in conclusion, familial periodic paralysis appears to be an abnormality associated with the mitochondrial elements and the endoplasmic reticulum in which there is an abnormality of carbohydrate metabolism and in which altered metabolism of the muscle cell in turn may produce the paresis by lowering the metabolic rate of the cell. It would appear from these recent studies that the serum potassium is merely a reflection of these profound intracellular metabolic events and that paresis may be severe and extensive in the presence of normal serum potassium or may be absent in the presence of abnormal serum potassium. Since by the Hodgkin-Huxley equations, the resting potential is $58 \times \log \frac{Ko}{Ki}$ (mV) and no hyperpolarization becomes apparent, then other cations and anions must shift during the period of paresis to account for the normal resting potential in such patients.

PARAMYOTONIA CONGENITA

(ADYNAMIA EPISODICA HEREDITARIA)

Eulenberg in 1886[4] presented a family which he traced through six generations in which the characteristics were as follows: (a) If an individual were affected the parent must always be affected. (b) The disease was characterized by myotonia, particularly of the facial muscles and of the tongue. Such a myotonia could, however, be more generalized. (c) The myotonia in this disorder, as in others was accentuated by cold. (d) The disorder was differentiated from other myotonic disorders by the fact that there were also episodes of severe flaccid paralysis. Eulenberg[4] pointed out that the giving of carbohydrates might abort the attack. He also pointed out that the disease was present from birth. In most neurological texts, this type of myotonica has been classified as a form of either myotonia congenita or dystrophia myotonia and the attacks of flaccid paresis and the genetic pattern of single dose trait are

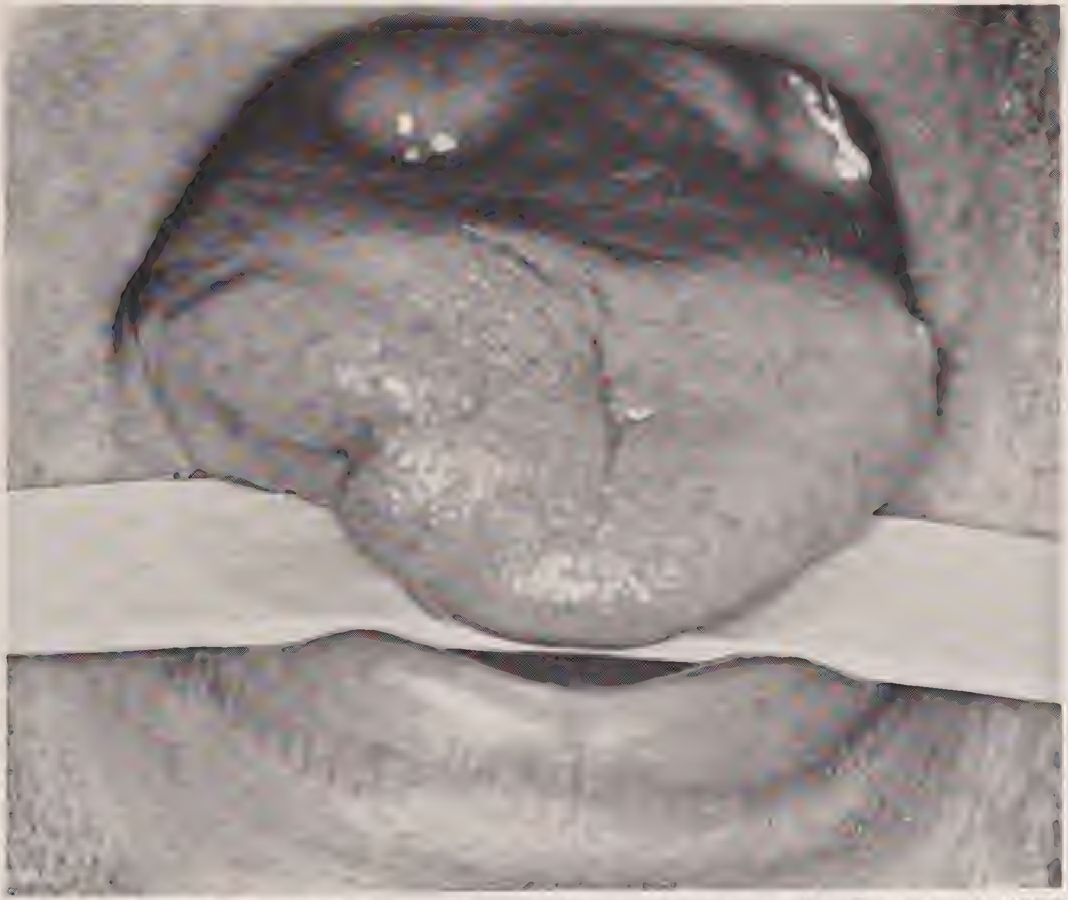


Fig. 11. Myotonia in the tongue in a patient with paramytonia congenita. Drager et al. (1958)

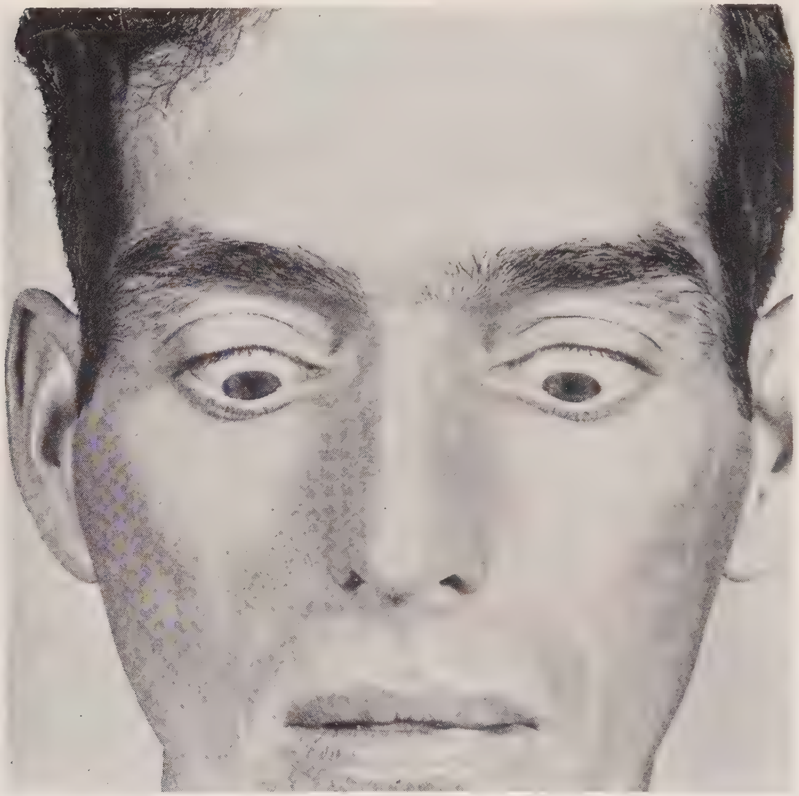


Fig. 12. Characteristic lid lag in a patient with paramyotonia congenita. Drager et al. (1958)

generally ignored. Aside from isolated descriptions of afflicted families, further progress in this disorder rested until 1956 when Gamstorp[6] described the disorder of hyperkalemic paralysis seen as a single dose trait in a large Swedish family. She termed this disorder *adynamia episodica hereditaria.* Although she did not find myotonia in this family, such patients did have a positive Chvostek sign, although they had a normal serum calcium. Since this report, many families[24, 13] have been described with hyperkalemic paralysis all of which, with the exception of Gamstorp's, have been associated with myotonia. Drager *et al.*[2] in the review of their particular family found many individuals in whom the myotonia was limited to the tongue (Fig. 11) or could be brought out in facial muscles only on the application of cold. They emphasized the phenomenon of lid lag (Fig. 12), and ascribed this to myotonia of the orbicularis oculi. McArdle[13], in his description, has emphasized a similar phenomenon. Drager *et al.*[2] on analysis of the potassium content of the muscle during the time of paralysis once again demonstrated little if any potassium change. Norris[15] has subsequently recorded intracellularly and found once again a normal resting potential during the time of the attack. Vacuolization of muscle fibers also have been found in this disorder during the period of the attack. Thus, in this particular disorder, it would appear that the opposite metabolic factors from familial periodic paralysis occur. Carbohydrate studies have not been done in such disorders but it would be of extreme interest to repeat the studies listed above for familial periodic paralysis. Once again there is no direct correlation between the potassium level and the amount of paresis and this would indicate that factors other than potassium are involved. In this disorder also, at the time of weakness, the deep tendon reflexes were significantly depressed in the involved muscle. Like familial periodic paralysis no equilibrium between potassium of the serum and muscle was present on the twenty-second hour. Unfortunately, basal metabolic rates were not obtained. Thus, familial periodic paralysis and paramyotonia cogenita, both of which may present identically with a flaccid quadriparesis, appear to be metabolically opposite diseases in which there is a hypokalemia in one and a hyperkalemia in the other. In neither of these is the potassium level correlated with the severity of the paresis.

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Disturbances of Electrolyte Metabolism in Selected Pediatric Problems

THOMAS E. CONE, JR., M.D.

INTRODUCTION

Three commonly encountered disturbances of fluid and electrolyte balance in pediatric practice are (a) hypernatremia as a result of severe diarrhea; (b) metabolic acidosis caused by severe and persistent vomiting, and (c) the effects of infectious diseases on body fluids and electrolyte balance.

HYPERNATREMIA

That hypernatremia may accompany severe diarrhea was first documented by Schmidt in 1850, but attention was not focused on this relationship until almost a century later[8]. In the past two decades, much has been learned about hypertonic dehydration (hypernatremia), but many questions remain unanswered. For the pediatrician, hypernatremia is synonymous with hypertonic dehydration because sodium salts represent almost all the solutes that usually contribute to the effective osmolality of the extracellular fluid.

The incidence of hypernatremia in moderate to severe diarrhea is variously reported as from 16 to 53 per cent; the latter figure was reported a few years ago from an epidemic in Minnesota. In this epidemic all the hypernatremic patients had received either (a) whole milk without added water; (b) milk plus Lytren, or (c) regular-strength Lytren* alone. On many occasions the latter two formulas were prepared incorrectly by the mother. All of the infants with normal or low serum sodiums either vomited their feedings or were given one-half strength skimmed milk, weak tea, carbonated beverages, or one-fourth strength Lytren. Infants on breast milk were less likely to develop hypernatremia than those fed the solutes from cow's milk, which presumably require four times the volume of water for excretion than does human milk. The well infant, in an environment which is not excessively hot, has enough renal capacity to cope with this added solute load, but with the added burden of water loss by way of the stools plus hyperventilation and skin loss secondary to fever, dehydration will ensue. This leads to a further decrease in renal reserve.

In general, patients with hypernatremia will show the following clinical features[11, 15]:

1. Most are under one year; beyond this age, other conditions are usually present and causative.
2. Most have *severe* diarrhea with anorexia and vomiting, and interference with oral intake of water[14].
3. Commonly, isotonic or hypertonic fluid therapy has been used. Water *loss* alone probably cannot cause serum sodium concentration of over 170 mEq. per l.
4. Renal or central nervous system diseases precede many of the cases, especially after one year of age.
5. All have central nervous system depression plus increased muscle tone, often with generalized rigidity and opisthotonus with increased deep tendon reflexes.
6. Cerebrospinal fluid protein is usually elevated (without pleocytosis), and the electroencephalogram is abnormal.
7. Signs of dehydration are deceptive, since dehydration is predominantly intracellular with a relative maintenance of the extracellular space.
8. Oliguria is the rule; but, despite dehydration, urine concentration is usually low. Renal clearances are initially very low with resultant azotemia.
9. The incidence of prematurely-born is high in some series[8, 11].
10. Mortality is significantly higher (up to 5x) in hypernatremic patients as compared to those with isotonic dehydration and with serum sodium concentration below 150 mEq. per liter.

In the *normal* subject, dehydration results in decreased renal sodium excretion and increased plasma sodium concentration in an attempt by the body to prolong survival time. The hypernatremia causes an increase in antidiuretic hormone activity and a decrease in urinary water loss. The higher osmotic pressure which results permits the maintenance of the extracellular and plasma volumes within fairly normal limits. Potassium also passes out of the cells and into the urine, producing a negative potassium balance. In hypernatremic infants, however, maximal urine concentration with potassium excretion does not occur early, and later, the capacity of the kidney to excrete water as well as electrolytes may be temporarily but seriously reduced. Renal immaturity may be involved, but body responses to the osmolar disturbances are also undoubtedly responsible for the kidney's inability to excrete excessive potassium. Hypothalmic influences are postulated by many workers, but cause and effect are hard to assess[15]. In addition, the *normal* infant is less able than the adult to excrete an exogenous sodium load, with the ceiling of excretion in the face of dehydration probably in the range of 5 to 10 mEq. per kg. per day.

Finberg[6] also proved that central nervous system signs and symptoms may be primary or secondary to hypernatremia. Injury to the brain, altering the regulatory mechanisms for homeostasis of water and electrolytes, may result in hypertonicity; also, decreased consciousness may produce decreased thirst, or dependent persons (such as infants) may not be offered sufficient water. Of course, central nervous system also may be *secondary* to hypernatremia: (a) Changes in the chemical anatomy of the cell and its environment necessitate osmotic readjustment by cellular water loss and a change in cellular constituents to increase osmolar concentration. Muscle cells take in sodium to aid in reaching this equilibrium, but the cells of the central nervous system do so minimally or not at all. Hyperosmolarity *per se*, without water shifts, as seen after the initial period in urea administration, does not seem to produce central nervous system symptoms[9]. (b) Changes in Na^+, $C1^-$, K^+ and Ca^+ concentration extracellularly can alter nerve cell irritability. (c) Gross central nervous system changes may occur, aggravating the already-existing symptoms resulting from hypertonic dehydration. (d) Dehydration itself may reduce the circulation of blood to the brain with concomitant serious physiologic alterations in brain function.

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*"Regular strength" Lytren contains 25 mEq. sodium and 25 mEq. potassium per liter.

McDowell *et al.*[13] state that, especially in the cells of the central nervous system, ions bound to protein may be made osmotically active, and/or the molecular arrangement of complex phosphates may be altered without disturbing the net electric charge. This production of what the authors call "idiogenic osmols" might disturb central nervous system function. It is not clear, however, whether these idiogenic osmols accumulate because sodium does not enter the central nervous system cell, or *vice versa*[9].

The skull being relatively nonyielding, the brain cell shrinkage, secondary to rapid and early loss of intracellular fluid, undoubtedly produces an increase in the blood and cerebrospinal compartments[4, 9]. The increased intravascular space probably results in the frequently-seen intracerebral and intradural hemorrhages, perhaps occurring in all patients with severe hypernatremia[9, 5]. In addition, brain shrinkage may cause tearing of the bridging veins in some patients, resulting in the formation of subdural hematoma[4, 9].

Hypocalcemia often accompanies hypernatremia; metabolic acidosis may protect against overt tetany *unless* the acidosis is corrected faster than is the hypernatremia. Tetany may occur early in the illness if only minimal acidosis is produced[11]. Renal shutdown with phosphate retention may aggravate hypocalcemia, but it almost certainly is not the primary factor. Finberg[7], in experiments with rats, found that hypernatremia produced hypocalcemia, but only in the face of an associated body-potassium deficit. Some have postulated that surfaces of bone crystals may act as a flexible sodium reservoir in electrolyte disturbances, and that increases in such sodium accumulation might interfere with the maintenance of calcium homeostasis.

Hypernatremia, then, must be suspected in any infant or young child with weight loss out of proportion to the signs of dehydration, especially in the presence of a "doughy" or "rubbery" feel to the skin, or with lethargy accompanied by irritability. Rehydration must be slow, with a minimal addition of sodium to the parenteral fluid early in therapy. Most workers recommend parenteral fluids containing from 15 to 30 mEq. Na per l. with necessary additional amounts to cover losses(stools, urine, and insensible loss) plus 10-20 ml. per kg. per day for depletion. Since no renal excretion of Na^+ or K^+ may occur for some time, and since oliguria is usually present, management must be essentially that of the patient in acute renal failure until proven otherwise. Added calories (glucose) decrease catabolism and, therefore, the amount of the metabolic solute load. In most patients, one-fifth to one-eighth normal saline in 5 per cent dextrose, started at about 100 ml. per kg. per twenty-four hours and adjusted as necessary, is advocated. Some recommend using Na lactate for a portion of the sodium concentration.

Too-rapid administration of water may produce water intoxication with seizures, but, with *careful* management, convulsions are probably the result of primary or secondary central nervous system changes as previously mentioned. Water intoxication, if suspected, should be treated with hypertonic saline; but, this should not be used in the absence of water intoxication. Paraldehyde or barbiturates are probably best for control of seizures secondary to the hypernatremic state itself. As previously mentioned, tetany can *usually* be prevented by avoiding too-rapid correction of the acidosis. Calcium gluconate I. V. should be used to treat tetany if it occurs (up to 2.0 ml. per kg., and a total amount of not over 20 ml., with slow injection and cardiac monitoring). One must stop with the first signs of bradycardia or changes in the electrocardiogram.

Some edema is to be expected in moderate to severe cases during rehydration and does not indicate a need to stop therapy. This edema is related to an overexpanded, extracellular fluid space, as intracellular fluid uptake lags behind. Patients should be followed very closely, with frequent weighing as well as serum electrolyte determinations. An attitude of watchful waiting should be adopted in reference to the central nervous system symptoms; a lumbar puncture may be done, but a subdural tap should not be performed unless there are real indications for such a procedure. Delay in therapy may result in irreversible, severe brain damage or death[11, 12].

METABOLIC ALKALOSIS CAUSED BY PERSISTENT VOMITING

Another common electrolyte disturbance seen among pediatric patients is metabolic alkalosis. This is usually caused by severe and persistent vomiting with a concomitant loss of hydrochloric acid of the gastric juice. Less often it may also be caused by the administration of excessive amounts of alkaline salts such as sodium bicarbonate, particularly in the presence of renal impairment. The main fluid therapy problem concerning vomiting in pediatrics is, of course, the therapy of hypertrophic pyloric stenosis.

Darrow *et al.*[5] have shown that alkalosis with hypochloremia may be associated with decreases of intracellular potassium and increases of intracellular sodium in infants with diarrheal disease and in rats made potassium-deficient by low potassium diets and injections of desoxycorticosterone. Such alkalosis may occur as a result of renal excretion of chloride without sodium as shown by Berliner[2], or of shifts of cations between muscle and extracellular fluid apart from renal mechanisms. The repair of such alkalosis following the administration of potassium is associated with a reversed shift of potassium and sodium between muscle and extracellular fluid.

Symptoms: Numbness, tingling sensations, headache, stupor and convulsions may be related to the state of alkalosis. Hypocalcemic tetany is usually not accompanied by alkalosis, but alkalosis, developing in a susceptible infant, may precipitate tetany. Alkalosis caused by persistent vomiting is associated with dehydration, whereas alkalosis caused by the excessive ingestion of alkalizing salts often may be associated with edema.

Low serum potassium concentrations may reflect the intracellular potassium deficiency, but the electrocardiogram may be even more useful. These patients frequently show the changes of hypokalemia: depression and broadening of the T wave and a prolonged Q-T interval.

In prolonged vomiting, the infant loses relatively larger amounts of chloride than sodium; for this reason, initial expansion of extracellular volume is best undertaken with isotonic sodium chloride rather than Ringer's lactate. Correction of the hypochloremia by administration of ammonium chloride without correction of the potassium deficit leads to continued alteration in the function of renal cells as well as other cells and subsequent difficulties in correction of the alkalosis. Although most of the deficits may be replaced within twelve hours, operation should be delayed for about forty-eight hours to permit further correction of the potassium deficit and allow for optimal readjustment of body functions, except in very mildly ill infants with little or no signs of dehydration.

FLUID AND ELECTROLYTE BALANCE IN INFECTIOUS DISEASES

Finally, a short discussion of the general features of infectious diseases and their effects on body fluid and electrolyte balance would seem appropriate[10]. Infections usually cause alteration of fluid and electrolyte balance when associated with excessive losses, as in diarrhea, when involving the central nervous system or when associated with limitations placed on normal homeostatic mechanisms, as in renal or pulmonary infections.

Common to most infections are fever and the metabolic reactions to stress. Multiple forms of stress, including infection, produce a relatively general metabolic pattern, characterized by dilutional hypotonicity, expansion of blood volume, fall in serum Na^+ and relative increase in serum K^+ and negative nitrogen balance.

Fever in infections is the resultant of two forces, heat production and heat elimination. Only in the presence of persistent, sustained hyperpyrexia can one say that basal heat production increases approximately 13 per cent for each degree C. rise in body temperature. Usually, if the increased heat production in infectious diseases can be met by adequate heat elimination, including insensible water loss, the need is for water alone, in addition to maintenance needs. But, when sweating occurs, appreciable losses of electrolytes take place and must be replaced.

Anorexia accompanied by vomiting is frequently observed during the course of infections in infants and children. These symptoms play an important additive role in relation of fluid and electrolyte aberrations. If these symptoms are present, maintenance needs, in addition to replacement of excessive loss of fluid and electrolyte, must be provided by the parenteral route. Fluids given to the dehydrated infant and child may be life-saving. Errors in therapy, however, can cause secondary injury or death. However, parenteral fluid therapy may be simplified so that most of the pediatric patients with fluid and electrolyte disturbances can be effectively handled by an empirical method based on body weight[1].

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Alterations of Serum Electrolytes in Burns

JENO E. SZAKACS, M.D., AND ELGIN C. COWART, M.D.

INTRODUCTION

Thermal trauma has plagued humanity throughout recorded history. The earliest medical scripts record the misery of the burned patient and suggest varied therapeutic procedures. The different approaches to therapy of burns have been reviewed by Cockshott[5]. Early attempts centered around relief from pain and local treatment of the wounds. Applications of light earths in vinegar and prescriptions for the use of ashed pigeons' dung mixed in oil were handed down from the Greek and Roman physicians.

Progress toward understanding the nature of physiopathologic alterations in burned patients was slow. In 1813, we find such enlightened observations expressed as that of J. Thompson, that "constitutional symptoms are generally rather proportionate to the extent than to the intensity of local affection" in the burned patient. At about the same time in Baltimore[5], carded cotton was introduced for dressing of burned wounds in much the same way as pressure dressings are applied today. The efficiency of this treatment, in the hands of Thompson and his contemporaries prior to Lister, compares favorably with our own, according to the analysis of Moyer[18]. Listerism led to indiscriminate applications of phenol, picric acid, medicated waxes, and eventually, to tannic acid to the burned surface, doubling and tripling mortality from burns as compared to the pre-Listerian era.

It was only after the turn of this century that supportive therapy in the form of saline infusions by all parenteral routes, and saline administration by mouth and by rectum was practiced by Sneve[25]. Sneve's results with the severely burned patient in 1905 were as good as the results in the years of 1944 to 1946 in America or England. It amounted to a mortality of 50 per cent in patients with thermal injury involving more than 25 per cent of the body surface area and aged less than sixty-one years. This therapy gradually gained support by studies such as Davidson's[10] observation on progressive fall in serum chloride, associated with reduced urinary excretion of this electrolyte in burned patients. Knowledge was later extended by a great many animal experiments. The reports are numerous and were surveyed in the Harkins[15] and Sevitt[24] monographs discussed at different Symposia on Burns[23, 28] and are presented in a number of original articles by Cope, Moore, Moyer, Evans and their colleagues, and others, between 1940 and 1950.

The report dealing with the Cocoanut Grove burns at the Massachusetts General Hospital[16] is a classic which included pathophysiologic observations on shock, metabolic studies, and a note on the blood bank in addition the surgical aspects of the care of the patients. By 1946, burn shock was reduced to 3 per cent as cause of mortality in the burned patient as opposed to 50 to 70 per cent, as estimated at the beginning of World War II. In the years 1942 to 1943, the Navy and Marine Corps fatality rate per hundred cases of extensive burns was 17.4. With better understanding of fluid and electrolyte therapy, the same group in 1944 to 1945 suffered only 5.9 to 7.4 fatalities per hundred[27]. An analysis of the cause of death in civilian population for pre-1946 and post-war periods is presented by Phillips. She also shows that electrolyte and fluid replacement therapy reduced mortality from shock to insignificant levels, but infection and respiratory damage currently cause 60 per cent of the deaths[19].

Probit analysis of mortality data recently published from Brooke Army Medical Center reconfirms other reports[20], that no significant gains in therapy were achieved during the post war period. Improvement in treatment may be achieved by combatting sepsis. Even more significant might be effective prevention of burns, as statistics now available show that 70 per cent of burns in children and 50 per cent in adults could have been easily prevented[1]. The need for prevention cannot be overemphasized. Peacetime accidents accounted for 7,300 deaths due to burns in the U.S. in 1960, and Pulaski[21] estimated the number of hospitalized burn patients at 30,000 per year. In war, since the discovery of gunpowder, burns became more frequent and more severe, and atomic warfare might surpass in extent all the greatest conflagrations ever known to humanity.

It can be concluded that the approach to therapy of the burned patient was an empirical art until World War II, without the benefit of thorough understanding of physiopathologic changes in the patient. The observation of Thompson, connecting severity of constitutional symptoms with the surface area burned, and the early supportive electrolyte therapy of Sneve now appear prophetic, and as such, they were not followed up with definitive studies until the 1940's. Modern understanding of the needs of the patient for fluids and electrolytes and the satisfaction of his nutritional needs is gratified by the radical reduction of mortality from burn shock.

THE BURN SYNDROME

Burn patients, in their altered physiologic reactions, manifestly follow a pattern regular enough to warrant the term "burn syndrome." Moyer states the "burn syndrome is characterized by alterations in fluid balance, larger than in any other injury, and the delayed closure of the wound." According to the operating pathologic and metabolic processes that dominate a particular period of the burn syndrome, four consecutive phases can easily be distinguished. Electrolyte shifts and massive fluid volume accumulation are observed in the first phase, and redistribution of burn edema predominates in the second phase. The last two phases are dominated by metabolic changes. A tissue catabolism occurs during the third phase from the tenth day until wound closure is completed. The fourth phase constitutes the phase of recovery and is characterized by positive nitrogen balance.

First Phase: The acute post-burn period, up to forty-eight hours, is characterized by increased capillary permeability and loss of vasmotor tone of terminal arterioles in injured tissues. This leads to accumulation of a massive protein-rich exudate in subcutaneous tissue and skeletal muscle underlying the burn, constituting wound edema. Accumulation of edema in the wound appears to be both obligatory and parasitic in the sense that it forms to the detriment of the unburned tissues. It expands the interstitial fluid space at the expense of plasma volume.

TABLE I BURN SYNDROME FIRST PHASE

First 48 hours post burn
Increased capillary permeability
"White bleeding" burn edema
Decreased plasma volume
Hyponatremia, hypochloremia
Hypoproteinemia
Oliguria, sodium and chloride retention
Burn shock

Accumulation of burn edema begins immediately after injury at a rapid rate. It seems to be triggered by extremely high osomtic pressure of burned tissue[1, 2], and it is maintained by elevated, arterial capillary pressure ahead of extensively leaking capillaries. The edema reaches its maximal volume at about forty-eight hours following injury. The amount of the edema depends not only on location of the burn and relative distensibility of tissues but also on the type of heat trauma. Scalding is known to produce the most impressive volume of edema. Flash burns are somewhat less, and deep flame burns with dry surface areas are least apt to produce massive edema. The therapeutic implications of this divergence in edema production and comparative electrolyte distributions were studied by Fox[14]. All types of burns are associated, however, with an obligatory edema that develops regardless of treatment or with the complete lack of it.

Capillaries lose their sieving properties during the first phase of the burn syndrome. They permit the leakage of protein, particularly albumin, while the larger molecules of globulins and blood corpuscles are retained in the vascular bed. This leads to hemoconcentration and to a transitory hyperproteinemia, at least theoretically. This causes resorption of interstitial fluid from unburned areas where a relative integrity of capillary wall is maintained. Plasma protein and sodium concentrations drop, and with continued losses to the wound, circulation fails, because of reduced plasma volume. Renal conservation of water and sodium tends to compensate for dehydration, and urine output is gradually reduced to complete anuria. Thus, the patient, several hours following the burn, if untreated, develops burn shock. Adults might be able to compensate for plasma volume losses provoked by burns involving up to 20 per cent of the body surface and might need only oral supportive treatment. Extensive burns require rigorous intravenous fluid and electrolyte replacement therapy. Less extensive burns in children, as in the aged, also require this therapy; increased mortality in these age groups is to be expected. An excellent illustration of mortality profiles recapitu-

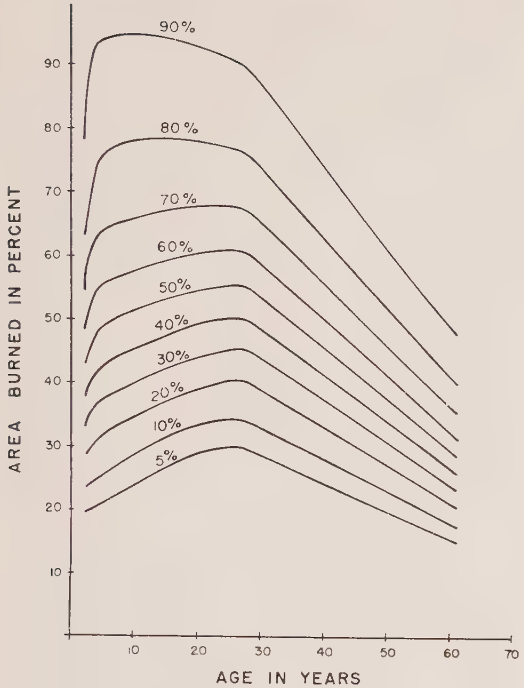

Fig. 1. Mortality contours of 1,100 burns treated at U.S. Army Surgical Research Unit, 1950-1960. Courtesy of Pruitt, B. A., Jr., Tumbusch, W. T., Mason, A. D., Jr., and Pearson, E.: Ann. Surg. 159:396-401, 1964.

lates the experience of the U. S. Army Surgical Research Unit[20] in Figure 1.

Quantitative Aspects of Fluid and Electrolyte Shift. The composition of the edema fluid was studied by collecting effluent lymph and bleb fluid for analysis. It was assumed that these samples are representative and both fully reflect the composition of the edema itself. Electrolyte distribution in these samples is the same as in plasma. The protein concentration, although elevated (4 to 4.5 gm. per 100 ml.), as compared to normal interstitial fluid values (1 to 1.5 gm. per 100 ml.), never reaches the concentration of protein in plasma. It reaches 60 to 80 per cent of the concentration of plasma albumin but only 40 per cent of the plasma globulin[7].

Cope and Moore[8] estimated in burned patients the variations in body fluid compartments, using thiocyanate and isotopic sodium procedures. These authors were able to chart expansion of extracellular fluid space against extent of the patient's burned surface area. While a linear relationship exists in the lower ranges, a maximal expansion of the extracellular fluid compartment is reached with burns affecting 50 per cent of body surface. This maximal expansion, compatible with survival, amounts to half of the normal extracellular fluid volume of the patient or approximately 10 per cent of the patient's body weight. In terms of solutes, in a 70 kg. man, a maximal burn edema (50 per cent surface area burn) would sequestrate the components:

7000 ml. of water	
1000 mEq. of sodium	(142 mEq. per L.)
750 mEq. of chloride	(107 mEq. per L.)
150 mEq. bicarbonate	(22 mEq. per L.)
350 gm. of protein	(5 grams per 100 ml.)

In order to prevent shock, this massive amount of water and the osmotically active colloids and sodium chloride need to be replaced. In addtion, water is necessary for urine formation for insensible water loss and for losses through the wound surface.

Since the losses are predictable on the basis of extent of the burn and the weight of the patient, prompt replacement therapy can be instituted. Useful as guide lines are the formulas devised by Cope and Moore, Evans, and Brooks Army Medical Center[3, 8, 11, 17, 22].

Plasma and dextrose are in general use to replace colloids. Both are effective to increase the circulating volume and to correct hemoconcentration. Both contain about 160 mEq. of sodium per liter. Balanced salt solutions, lactated Ringer's and Haldane's solutions, are designed for electrolyte replacement. Potassium is not replaced specifically. There is experimental evidence that in animals, potassium replacement in the first phase is not only useless but dangerous[17]. Fox has shown that potassium is released from burned tissue, and it is replaced quantitatively by sodium[13], but significant elevation of potassium concentration in plasma occurs only with very extensive and deep burns in the absence of renal disease. Low normal values are seen at times, particularly if diarrhea is present. The rate of fluid replacement should keep step with edema formation, rapid at first, then tapering off gradually. Adjustments in the rate of infusion are best gauged to the hourly output of urine. Urine flow should be maintained between 25 and 50 ml. per hour. The presence and extent of respiratory damage due to hot gases calls for great caution, as massive pulmonary edema might develop during fluid replacement[19].

The most comprehensive recent study of the pathophysiology of the first phase by Arturson[2] reconfirms much of the earlier reports by Field[12] and Cope[7, 8, 9] and their colleagues. Arturson extended earlier observations and was able to document with his technique of dextrane infusions a generalized increase in capillary permeability following extensive scalds and, particularly, increased renal glomerular permeability in burned patients.

In conclusion, the first phase shows massive sequestration of all plasma electrolytes, protein, and water in the burn edema. The burned tissue itself exchanges potassium for an equivalent amount of sodium ions; there is marked decrease of plasma sodium, chloride and protein concentrations and tendency for increased renal retention of water and electrolytes.

Second Phase: The second phase is characterized by massive fluid and electrolyte redistribution. From the third to the eighth day of post-burn, edema is reabsorbed via the lymphatics at a somewhate variable rate, and diuresis begins, often massive, in the face of radically reduced intakes of fluid and electrolytes. Large amounts of water are excreted obligatorily with the nitrogenous products of cellular catabolism and nitrogenous products of tissue necrosis. A relative retention of sodium may result, which is recognized by increased concentration of sodium in serum. The relation of concentration of serum sodium to hydration of the patient in this period was extensively investigated by Saroff and his colleagues at Brooke Army Medical Center[26]. They found that concentrations of serum sodium between 130 and 140 mEq. per L. are characteristic of adequately treated patients. Increasing concentration of serum sodium is associated with reduced urinary output and is a sign of cellular dehydration. Patients exhibiting progressively increasing sodium levels (positive balance) were found to receive, during their fluid therapy, significantly less "available non-electrolyte water" than the patients without change in sodium concentrations. The largest amounts of "available non-electrolyte water" were found in patients with progressively decreasing sodium levels (negative balance). In the second phase of the burn syndrome, the concentration of serum sodium is the most sensitive index of the patient's status of hydration.

Table II Burn Syndrome
Second Phase

Third to eighth day post burn
Redistribution of water, protein and electrolytes
Diuresis, relative sodium retention
Wound exudate losses
Complications: Hemoglobinuric nephrosis
  Infection, Septicemia, Intoxication
Anemia

Water with 5 per cent dextrose should be administered during the diuresis gauged to maintain sodium levels at 135 mEq. per L. of plasma. Potassium, during the second phase, might be in balance if the patient is able to eat. A slight negative balance might prevail until the tenth day, thereafter paralleling the nitrogen balance curve. Starting on the third day of post-burn, a negative potassium balance should be corrected to avoid a marked drop in potassium concentration. An intake of 80 to 100 mEq. of potassium per day is adequate. In this phase, protein catabolism exceeds two or three times the protein intake, and besides potassium, other intracellular electrolytes are lost excessively, such as magnesium, sulphur, and phosphorus. The patient is febrile as a rule, and infection and septicemia are the major threats which remain until closure of burn wounds. During this period, anemia might develop, and supportive therapy with blood might be necessary. Hemoglobinuria is a rare complication of the first two phases, and, unless preexisting renal disease interferes, the diuretic phase allows for excretion of mobilized sodium and chloride from the burn edema.

Exudate losses from the burn wound are significant and might account for as much as 3,000 ml. of protein rich fluid per day. Loss through the wound is particularly important in children, as is pointed out by Batchelor and his colleagues[4].

Third Phase: The third phase is characterized by a general exhaustion of the patient and a minimum resistance to stress. Catabolism with negative nitrogen balance continues until wound closure is complete. In this period, from tenth to approximately thirtieth day of post-burn, complete supportive therapy, by diet, parenteral replacement, antibiotics, and skillful care for the wound, is imperative. This is the most difficult period in burn management. The negative balance of all intracellular electrolytes, protein, fat, and carbohydrate, places great emphasis on metabolic studies and search for palatable high caloric diets. Slight acidosis might be present in uncomplicated cases due to lactic acid production, but actual pH measurements are rarely necessary. Careful charting of the patient's weight is one of the most informative procedures. The laboratory support for the burned patient, besides the role of the blood bank, is accomplished by relatively few tests. Hematocrit measurements and analysis for serum sodium are the most important.

Table III Burn Syndrome
Third Phase

Eighth to thirtieth day post burn
General exhaustion with incipient repair
Excessive sensitivity to stress
Wound exudate losses
Negative nitrogen and electrolyte balance
Renal, pulmonary or cardiovascular complications
Atrophy of most organs

Fourth Phase: The fourth phase, or recovery, marks positive nitrogen metabolism with the wounds covered and healing. Electrolyte disturbances are not significant at this stage.

TABLE IV BURN SYNDROME
FOURTH PHASE

Wound closure complete
Positive nitrogen balance
Recovery complications: Serum hepatitis

CONCLUSION

In conclusion, the burned patient follows a well delineated pattern in his deranged physiology. Electrolyte and fluid accumulation in the burned tissue is obligatory at first and may lead to circulatory failure and shock. Redistribution of electrolytes and water dominates the second, or diuretic phase, of the burn syndrome. A slow, insistent drain of electrolytes, protein, and caloric energy is characteristic of the third phase. Quantitation of these changes, and a better understanding of the local phenomena in the burn tissue and its effect on the host, is the most significant modern development in burn physiology.

Modern fluid and electrolyte replacement are the most significant improvement in burn therapy, though only a partial victory for medicine, as the patient saved from shock is still subject to the ordeals of sepsis and the not-yet-completely-understood catabolic phase of the burn syndrome.

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Alterations of Serum Electrolytes in Intoxication with Salicylates, Bromides, and Methanol.

EARL B. WERT, M.D.

SALICYLATES

Nine million pounds of aspirin are consumed in the United States each year, with about two hundred fatalities reported annually (about 75 per cent of these being in children under four years of age). The subject of salicylate poisoning is of obvious importance.

Therapeutic doses of salicylate produce definite changes in the electrolyte and pH pattern of the blood, proportional to the dose and increasing with the period of medication. Toxic levels are considered to be about 9 mg. per 100 ml. of serum in children and 35 mg. per 100 ml. in adults. Sixteen mg. of salicylate per 100 ml. will increase the sensitivity of the respiratory center 100 to 300 per cent producing the initial respiratory alkalosis. Characteristically, the respiratory volume is increased by 4 liters per minute due to increased amplitude without increased rate. The CO_2 tension is decreased approximately 10 mm. of mercury; the bicarbonate is increased 3 millimoles per L.; and the plasma pH is increased by about 0.06 units.

The pathologic physiology of salicylate poisoning may be described under three headings: (a) The *stage of respiratory alkalosis,* caused by increased sensitivity of the respiratory center; (b) the *stage of metabolic acidosis,* caused by unknown mechanisms, but not by the salicylate ion *per se;* and (c) *other manifestations.*

1. The *stage of respiratory alkalosis,* resulting from salicylate effect on the respiratory center, increasing the sensitivity of the center to the carbon dioxide/pH ratio, usually raises the pH to 7.46 to 7.60.

Hypokalemia is an important consequence of respiratory alkalosis. While well-documented in metabolic alkalosis, hypokalemia is not commonly appreciated in respiratory alkalosis, but is seen in the severe and prolonged stress of salicylate intoxication. The mechanism for the development of hypokalemia is as follows: (a) An alkaline pH tends to shift potassium out of the extracellular compartment and (b) may deplete the body potassium by excess renal loss. Further, (c) salicylates have a direct effect on renal tubules and increase renal excretion of potassium, and (d) there is a mutual aggravation of alkalosis and potassium depletion. That is, with the stimulation for the renal excretion of potassium, hydrogen ions enter the intra-cellular compartment, increasing the extracellular alkalosis and producing potassium depletion, thus completing a circle. It should be noted that with adequate potassium replacement the second stage of salicylate derangement, that of metabolic acidosis, may be avoided.

2. The *stage of metabolic acidosis,* following quickly and suddenly stage 1, is not clearly understood, and cannot be accounted for entirely by the salicylate ion, ketosis, or renal failure. The suggestion has been made that this stage is indeed a respiratory acidosis due to increased CO_2 production. Salicylates interfere with oxidative enzyme systems, but detailed explanations are not available.

3. *Stage* 3 is marked by fever, hypoprothrombinemia, hemorrhagic gastritis, hypoglycemia, increased rate of metabolism, and renal changes. The renal changes are not marked except for increased release of potassium and for the inhibition of the tubular resorption of urates. Small doses of salicylates produce urate retention; larger doses result in their excretion. In fact, the concentrations of uric acid in serum are reduced so low and so consistently that this should be used as an additional diagnostic determination in suspected salicylate poisoning. In acidosis, free salicylate excretion may be increased by the administration of 5 mg. per Kg. of Acetazolamide every five to six hours. Potassium replacement may be necessary. Artificial dialysis has proved life-saving in certain instances.

BROMIDE

Bromide is rapidly absorbed from the intestinal tract and is distributed in the body similarly to chloride. The bromide space is practically the same as the chloride space, as determined by Gamble in 1953, using radioactive halides. Like chloride, bromide does not fully penetrate cell membranes, except for the erythrocytes which are permiable to both ions. Since chloride remains quite constant under normal conditions, and since the body does not readily distinguish between chloride and bromide, the same adjustments are made when bromide is administered. That is, excess halide is excreted. Thus, when bromide and chloride constitute the halide content of the body fluids their ratio depends on the intake and excretion of each ion. For example, bromide displaces chloride, and a considerable decrease in chloride may occur. The ratio of bromide to chloride is the same in extracellular fluid and serum, but there is less bromide in cerebrospinal fluid and brain than other body tissues. The kidney attempts to keep the halide compartment of extracellular fluid at a normal concentration but preferentially absorbs bromide and excretes chloride.

Bromide is very slowly eliminated. When 1 gm. of sodium bromide is ingested, 1 gm. of halide is excreted, low in bromide. Several weeks are required to excrete the ion completely. If bromide is administered steadily, a steady state is reached. For example, when 6 gm. per day are ingested, equilibration is reached in about two weeks, depending on the chloride intake. The less the chloride intake, the greater the bromide retained, and conversely, if the chloride intake is increased, bromide excretion is increased. This fact is the basis for the clinical treatment of bromide intoxication employing chloride. Further, in patients from whom chloride is withheld (e.g., cardiacs), larger amounts of bromide will be retained. Thus, ordinary doses of bromide may result in toxic levels, since the body will retain bromide in lieu of chloride.

The high incidence of bromism in the United States was first described by Wuth in 1927. In spite of numerous papers warning of the danger, bromism is common. Recently 10 per cent of admissions to a psychiatric hospital were found to have toxic levels of bromide. The toxic effects of bromide are seen at levels as low as 50 mg. per 100 ml. of serum, a fact not commonly appreciated. Levels of 75 mg. are suggestive and above 200 mg. are considered toxic.

METHYL ALCOHOL

After absorption, methyl alcohol is widely distributed in body fluids, being found in somewhat higher concentrations in cerebro-spinal fluid than in the blood serum. It owes its major, untoward effects to its oxidation products, formaldehyde and formic acid, and the acidosis produced. Minor factors are those of central nervous system depression and a specific, toxic effect on the retinal cells, probably by formic acid. In this regard Roe in 1948 pointed out that the oxygen requirement of the retina in relation to its iron-containing enzyme is greater than that of any other tissue, and he suggested that formic acid causes retinal hypoxia by specific inhibition of this enzyme, since formic acid combines with ferric and ferrous ions. As little as 4 ml. of methyl alcohol have produced blindness, and as little as 6 ml. of methyl alcohol have produced death. Death is always preceded by blindness in humans, an effect not seen in laboratory animals. Recently, an individual drank 540 ml. of methanol and survived.

The unpredictability of the effects of methanol are not entirely due to individual susceptibility. The variables are as follows: (a) The impure product may contain furfural, methyl acetate, dimethyl acetate, ethyl dimethyl ketone, and allyl alcohol. (b) Individual variation exists in the ability to oxidize methyl alcohol, a prominent finding in experimental animals. (c) The oxidation of methyl alcohol is inhibited by ethyl alcohol. This has been demonstrated by Roe and Zatman, who prevented methyl alcohol oxidation *in vitro* by equimolar concentrations of ethyl alcohol, and further, by demonstrating that the urinary excretion of methanol is greatly enhanced by ethanol, indicating diminished oxidation. Bartlett confirmed this in intact rats and liver slices using C^{14} labeled methanol.

The electrolyte changes are those of a metabolic acidosis. The blood pH is appreciably lowered, the CO_2 combining power is reduced to less than 20 volume per cent in fatal cases, and may be reduced to zero. Dramatic clinical improvement (including eye symptoms) follows the ingestion of alkali (6 to 8 gm. of sodium bicarbonate by mouth or one-sixth molar lactate in amounts sufficient to restore the CO_2 combining power to normal). Alcohol, by mouth or intravenously in 5 per cent glucose, will retard the rapid oxidation of methanol, allowing its excretion as methanol by lungs and kidneys. The ketonuria and acetonuria are relatively moderate in contrast to the severe acidosis that is present. The latter is due to the acid substances introduced, 25 per cent of which are formic, lactic, and acetoacetic acids, while 75 per cent are acids which have not been identified.

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Index

A

- Acetaldehyde, p-hydroxydiphenyl, condensation product with in lactate detn., 107, 108
- Acetate buffer, P detn. with, 98
- Acetazolamide, salicylate intoxication therapy with, 403
- Acetic acid
  - (1,2-cyclohexylene-dinitrilo) tetra-, Ca detn with, 38, 40, 44
  - [ethylenebis (oxethylenenitrilo)] tetra-, Ca detn with, 38
  - (ethylenedinitrilo) tetra-
    - alkaline soln. for Ca detn., prepn., 52
    - alkaline soln. for K detn., 33, 34
    - Ca detn. with, 37-42, 44
    - interference in Cl detn., 84
    - Mg detn. with, 57
    - renal tubular vacuolization due to, 310
    - Sr salt, in Mg detn. by flame photometry, 57
    - So_4 and PO_4 interference elimination with in Ca detn., 204
  - (methoxyphenyl)-, Na salt, Na detn. as, 9, 10
  - K precipitation with and precipitate solubility in presence of, 21, 22
  - potassium sodium cobalti-nitrite precipitate increased solubility in, 21
  - trichloro-, blood protein precipitation with, 23, 34, 91, 92, 101, 201, 202, 204, 207
- Acetoacetic acid
  - accumulation in ketoacidosis, 105
  - ethyl ester, standard soln. for ketone body detn., 110
  - level in blood and urine, value range, 112
  - thermal decarboxylation to acetone in ketone body detn., 109-112
- Acetone
  - condensation product with salicylaldehyde, 110
  - dihydroxybenzal-, formation, 110
  - in fatty acid detn. by gas chromatography, 115
  - ketone body detn. as, 109-113
  - sulfate detn. with, 94
  - total base detn. with, 163, 164
- Acetone bodies, *See* Ketone bodies
- Acetylcholine, release, Ca versus Mg effect on, 248
- Acid-base balance
  - acute disturbances, body fluid pH differentials in, 265-272
  - assessment, arterial vs. venous blood in, 345-347
  - clinical considerations, 135-137, 155
  - detn., serum CO_2 detn. in, 124
  - drug distr. in, 270
  - in liver disease, 353-360
  - in pathological states, 104-106
  - in renal failure, 106
  - K level control by, 20
  - Siggaard-Andersen nomogram, 133, 135, 136
  - Singer-Hastings nomogram, 133
  - Van Slyke-Sendroy nomogram, 133, 134
- Acidimetric method for Na detn., 10
- Acidity
  - hydrogen ion activity as expression of, 143
  - molybdate method for P response to, 97, 98
- Acidosis
  - in alcohol intoxication, 105, 106
  - anesthesia depth, response to, 341
  - in Asiatic cholera, 280
  - blood pH values in, 155
  - blood and cerebrospinal fluid pH shifts in, 265, 266, 267
  - body fluid compartment pH changes in, 265-272
  - in burn syndrome, 399
  - Cl balance in, 308
  - diabetic
    - electrolyte water loss in, 362-366
    - hypokalemia in, 29
    - serum total base values in, 161, 162
    - treatment evaluation, 105
  - hypokalemic, renal lesions in, 316, 317
  - lactic, serum lactate level in, 104, 105
  - in low intestinal obstruction, 334
  - metabolic
    - in hypernatremia of children, 390, 391
    - in methanol intoxication, 404
    - in salicylate poisoning, 402, 403
  - metabolic and respiratory, pCO_2 deviations in, 135, 137, 138
  - nephritic, serum total base values in, 161, 162
  - in renal failure, 106
  - renal tubular
    - K depletion in, 29, 312
    - renal parenchymal calcification in, 321
  - in salicylate intoxication, 105
  - serum K level and, 35
- Acids
  - aromatic, gas chromatography in urine, 113
  - keto, serum level, diagnostic significance, 104, 105
  - level in blood, normal values, 4, 5
  - non-volatile, accumulation, metabolic acidosis due to, 136, 138
  - organic
    - detn. in serum, general aspects, 104-118
    - elimination in acidosis, 136

        excretion in lobar pneumonia, 350
        excretion in K depletion, 318
        level in interstitial fluid in ketoacidosis, 105
        level in serum, in renal insufficiency, 300, 301
        level in serum, reported values, 104, 105, 159
        loss during tissue catabolism, 297
    total, reported values, 159
Acid tartrate, alcoholic soln., K detn. with, 23
Acromegaly, Ca and P metabolism in, 243
Adams-Stokes syncope, in aldosteronism, 230
Addison's disease
    hyperkalemia in, 29
    serum total base values in, 161, 162
Adenine, Na-dependent transport, 233
Adenosine, Na-dependent transport, 233
Adenosine diphosphate, cell respiration control by, 240
3,5 Adenosine monophosphate, role in glucose formation in familial periodic paralysis, 379, 380
Adenosine triphosphatase, active transport control by, 240
Adhesions, intestinal obstruction due to, 332
Adie-Wood method for K detn.
    modifications, 20, 21-31
    reported reactions, 20
Adipate, chromatographic column coated with for fatty acid detn., 114
Adrenal gland
    cortex
        adenoma, in aldosteronism, 229
        Ca and P metabolism control by, 244
        function, serum K level and, 29, 35
        hormones, K balance regulation by, 20
        hyperfunction, renal lesions in, 319
        hyperplasia in aldosteronism, 229
        insufficiency, serum electrolyte patterns in, 300, 301
    Na and K metabolism regulation, 228-232
Adrenalin, *See* Epinephrine
Adrenocorticotropin, aldosterone secretion and, 228, 229
Adynamia episodica hereditaria, characteristics, 386
Aeration apparatus, for NH_3 detn., 121
Alanine
    phenyl-, Na-dependent transport, 233
    Na-dependent transport, 235
Albanese-Lein method for Na detn., 11, 15, 18
Albumin
    base-binding capacity, 114
    loss in burn trauma, 396
    Mg binding power, 58
Alcock-MacIntyre-Raddle method for Mg detn., 57
Alcoholism
    liver dysfunction in, 353-360
    lowered serum Mg in, 250
    with terminal uremia, serum composition in, 302
Alcohol wash solution, for serum Na detn., prepn., 14
Aldehydes, long-chain, gas chromatography, 113
Aldosterone
    familial periodic paralysis and, 377, 378
    isolation and elucidation, 228
    secretion
        factors conditioning, 339, 340
        pitressin effects on, 277
        renal regulation, 228
Aldosteronism
    lowered serum Mg in, 250
    manifestations, 229, 230
    K depletion due to, 312
    K losing nephritis and, 318
    selective, manifestations, 230
Alkali metals, level in feces vs. plasma in cholera, 282
Alkaloids, gas chromatography, 113
Alkalosis
    blood pH values in, 155
    body fluid compartment pH shifts in, 265-272
    hypokalemic, in aldosteronism, 229
    metabolic
        in children, due to persistent emesis, 391
        in children, ecg in, 392
    metabolic and respiratory, pCO_2 deviations in, 135, 137, 138
    in K depletion, 318
    respiratory
        induction in anesthesia, 345
        in passive hyperventilation, 341
        in salicylate poisoning, 402
Aluminum, level in bone, 368
Amberlite IR-112, Na detn. with, 10
Amberlite IR-400, OH form, Na detn. with, 10
Ames-Sakanoue ultrafiltration apparatus, fig. and commercial source, 66, 67
Amin method for Ca detn., 42
Amino acids
    active transport, ATPase role in, 240
    level in serum, diagnostic significance, 104
    separation by gas liquid chromatography, 117
    Na-dependent transport, 233
    S-containing, S utilization from, 260
Aminonaphtholsulfonic acid reagent, prepn., 61, 101, 102
Ammonia
    detn. in serum
        apparatus, 120, 121
        calculations, 122
        error sources, 122
        general aspects, 119
        principle, 119

    procedure, 121, 122
    reagents, 119, 120
  intoxication, in liver disease, 356, 357
  level in serum, normal values, 123
  stability in fresh-drawn vs. frozen blood, 119
Ammonium carbonate, sodium detn. with, 8, 9
Ammonium chloride
  body fluid compartment pH shifts due to infusion of, 265-270
  infusion, hypochloremia correction with in metabolic alkalosis of children, 392
Ammonium hydroxide
  soln. for Ca detn. by Clark-Collip method, 48
  soln. for Mg detn., prepn., 60, 61
Ammonium molybdate
  P detn. with, 98
Ammonium molybdate reagent, for Mg detn., prepn., 61, 101
Ammonium nitrate, sulfate detn. with, 90
Ammonium oxalate, soln.,
  for Ca detn. by Clark-Collip method, 48
  for Mg detn., prepn., 60
Ammonia purpurate, *See* Murexide
Ammonium salts, cobalti-nitrite reagent reaction with, 20
Ammonium sulfate
  excretion, values, 263
  N standard soln. prepn. from NH_3 detn., 120
Ammonium thiocyanate
  K detn. with, 23
  Na detn. with, 11
  soln., for Cl detn., prepn., 82
Analgesics, respiratory response to in anesthesia, 343
Anast method for Mg detn., 57
Anderegg, cresophthalein complexon introduction, 41
Anderson-Weinbren method for Ca and Cr, 42
Andreason method for Mg detn., 57
Androgens, Ca and P metabolism response to, 244
Anesthesia
  acid-base balance in, 270
  blood and cerebrospinal fluid pH discrepancies due to, 266
  body habitus variations in, 337, 338
  body position effects in, 343, 344
  controlled respiration in, 344
  electrolyte metabolism in, 337-348
  respiratory acidosis in, 137
  respiratory exchange in, 341, 345
  ventilatory abnormalities in, 342
Anesthetics
  general, osmoregulatory system response to, 275
  volatile, serum CO_2 false values due to, 127
Angiotensin, aldosterone secretion regulation by, 228, 229
Anions, level in serum, reported values, 4, 5, 159
Anorexia nervosa, serum electrolyte patterns in, 293-296, 298
Anorexia, in hypernatremia of children, 389
Anoxia,
  hyperkalemia in, 29, 35
  serum lactic acid level elevation in, 105
Antidiuretic hormone
  formation vs. secretion abnormalities, differential diagnosis, 276, 290
  in heart failure manifestations, 290
  renal response to, review, 275
  secretion
    abnormalities, 276-279
    in acute intermittent porphyria, 278
    in anesthesia and narcosis, 337
    in hypernatremia of children, 389
  water metabolism regulation by, general aspects, 274, 275
Anuria
  hyperkalemia in, 29, 35
  serum composition in, 302, 303, 307
Apiezon L, Celite column coated with for fatty acid chromatography, 114
Argentimetric method for Cl detn.,
  evaluation, 83-86
  serum adaptation, 81, 82
  tissue adaptation, 82, 83
  urine adaptation, 82, 83
Argon, as carrier gas for serum fatty detn., 114
Arsenic
  detn. by X-ray spectroscopy, 212, 213
  molybdic acid complex, absorption spectrum, 98
Arsenite-citrate reagent, for ascorbic acid-reduced phosphomolybdic acid complex, 99
Arsine generator, in sample prepn. for X-ray spectroscopy, 213
Arterioles, terminal, properties in burn syndrome first phase, 396
Arteriosclerosis, serum fatty acids in, 117
Arteritis, necrotizing, in NaCl intoxication, 319
Ascites, in liver disease, 354-360
Ascorbic acid
  level in serum, diagnostic significance, 104
  as reducing agent for P detn., 99
Asia, southeast, diarrheal syndromes, physiologic aspects, 280-287
Asthma, anesthesia in, 342
Astrup's interpolation method for pCO_2, logarithmic plot, 130
Atropine, respiratory response to in anesthesia, 342, 343
AutoAnalyzer
  Ca detn. with, 38, 40, 41
Azotemia, in liver disease, 354, 356-358

B

Baar method for Ca detn., 37
Baar method for Cl detn., 76
Bachra-Dauer-Sobel method for Ca detn., 38
Bacteria, SO_4 producing, as S source, 260
Baginski method for Cl, detn., 76, 77
Ball method for Na detn., 10, 11
Barber-Kolthoff method for Na detn.
  Sunderman modification, 13-15
  uranyl zinc sodium reagent, 8
Barbiturates
  osmoregulatory system response to, 275
  seizure control with in hypernatremia of children, 391
Barbituric acid, 5-isonitroso-, *See* Violuric acid
Barium, removal for Na detn., 8
Barium chloride
  Na detn. with, 10
  SO_4 detn. with, 90-92
Barium chloranilate method for SO_4, general aspects, 92
Barium hydroxide
  -zinc sulfate combination
    body fluid deproteinization with for ketone body detn., 109
    protein precipitation with for lactate detn., 107
Barker method for lactic acid detn.
  error sources, 108
  principle, 107
  procedure, 107, 108
  reagents, 107
Barkla, X-ray emission studies, 209
Barney-Bertolacini method for Cl detn., 76
Barry-Rowland method for K detn., 25
Bases
  level in serum
    in fever and hypothermia, 296-298
    in renal insufficiency, 299, 300
  protein, reported values, 159
  total, detn. by chemical methods, 157-165
  total, detn. by conductivity methods, 166-175
  total, detn. by Hald benzidine method
    apparatus, 163, 164
    error sources, 165
    principle, 163
    procedure, 164
    reagents, 163
  total, detn. by Sunderman conductivity method
    apparatus, 173, 174
    calculations, 174
    clinical aspects, 161
    normal values, 174
    principle, 172
    procedure, 174
    reagents, 172, 173
  total, level in serum
    abnormal values, 161, 162
    normal values, 4, 5, 157-161
    in starvation, 294-296
    in uremia, 302
  total, values
    variability, 157-159
    for adults vs. children, 158, 159
    food intake effect on, 159
    in lobar pneumonia, 351
Bates-Guggenheim pH values, 144, 151, 152
Beck, N-hydroxynaphthalimide reagent for Ca detn., 51
Beckman method for freezing point detn., 176
Beckman Research pH Meter, circuit diagram, 154
Benzene, bromo-, S conjugation with, 261
Benzidine hydrochloride, standard solns. for SO_4 detn., 95
Benzidine method for SO_4
  calculation, 95
  general aspects, 94
  innovators, 90, 91
  procedure, 95
  reagents, 94, 95
  urine adaptation, 96
Benzidine-naphthoquinone-4-sulfonate derivative, spectral characteristics, 92
Benzidine reagent
  for SO_4 detn., prepn., 94, 95
  for total base detn., 163
Benzidine sulfate
  precipitate, diazotization in SO_4 detn., 91
  SO_4 detn. in, 90
  titration for total base detn., 165
Berzelius, electrolyte studies on muscle, 3
Bessman-Anderson method for ketone bodies, 109-112
Bett-Fraser method for Ca detn., fluorometric modification, 39
Bicarbonate
  level in blood, clinical considerations, 135-137
  level in edema fluid in burn trauma, 397
  level in serum
    in fever and hypothermia, 296, 298
    hyperventilation as decreasing, 341
    reported values, 4, 5, 159
    in salicylate poisoning, 402
    in starvation, 295, 296, 298
    in uremia, 302
    and relation to carbonic acid level and pCO_2, 131-134
  loss, in diabetic acidosis, 363, 365
  renal transport impairment in K depletion, 318
  shifts, Cl transport and, 254, 255, 257
  transfer among body fluid compartments in acid-base disturbances, 265, 266-270
  ubiquitousness in animal bodies, 3

- Bile, Cl transport to gastric juice, 256
- Bile pigments
  - destruction in icteric serum, 75
  - interference in Cl detn., 75
- Bilirubin, interference in Mg detn., 64
- Biological fluids
  - osmolality, detn.
    - by freezing point method, 176-181
    - survey of methods, 183
- Biopsy, percutaneous, in hepatic regeneration assessment, 360
- Bismuth
  - detn. by X-ray spectroscopy, 218
  - electroplating for X-ray spectroscopy, 214
- Blindess, in methanol intoxication, 404
- Blood
  - arterial, CO_2 tension, alveolar CO_2 tension and, 341-342
  - arterial vs. venous, in acid-base balance assessment, 345-347
  - branched-chain fatty acids in, chromatographic detn., 117
  - CO_2 level, serum pCO_2 detn. from by interpolation, 128-134
  - pCO_2, clinical considerations, 135-137
  - collection and prepn.
    - for NH_3 detn., 121
    - for CO_2 detn., 125, 127, 140
    - for pH electrometric detn., 150, 151, 154
  - electrolytes in, Greenwald composite method for detn. of, 22
  - electrolyte and water exchange with interstices, 6, 7
  - pH
    - clinical considerations, 135-137, 155
    - detn. by electrometric method, 143-156
    - serum pCO_2 detn. from by interpolation, 128-134
    - temperature-dependence, 147
  - Fe level in, distr. and values by X-ray spectroscopy, 226
  - ketone bodies in, detn., 109-111
  - lactic acid detn. in, Barker method, 107, 108
  - organic constituents, wet ashing in Clausen method for K detn., 22
  - K detn.
    - in Clausen cobalti-nitrite modification, 22
    - initial attempts, 22
  - K level, normal values, 20
  - SO_4 detn. in, historical account, 90
  - SO_4 level, 90, 91
  - S in, forms and values, 262
  - volume
    - factors conditioning, 337, 338
    - low, aldosteronism in, 230
  - Zn level in, clinical interpretation, 226
- Blood coagulation, Ca role in, 242
- Blood gases
  - drug effects on in anesthesia, 342, 343
  - gas chromatography, 113
  - status, in anesthesia, 341-347
- Blood plasma
  - alkali metals in, level in cholera, 282
  - cation levels in diarrhea, 283-286
  - deproteinization for ketone body detn., 109
  - electrochemical gradient with cells, Cl shift and, 255
  - expanders, in liver disease, 359
  - infusion, in burn trauma, 397
  - osmolality
    - in anesthesia, 341
    - regulation, 273-275
  - K level, normal values, 20
  - SO_4 level, 91
  - volume
    - in alcoholic cirrhosis, serum Na relationship with, 355
    - body habitus and sex related, 337, 338
    - in burn trauma, 396
    - in liver disease, paracentesis effect on, 357
  - Zn level in, values by X-ray spectroscopy, 226
- Blood pressure, anesthesia depth response to, 341
- Blood proteins
  - base-binding values, 160
  - Ca detn. in presence of, 42
  - digestion for X-ray spectroscopy, apparatus for, 213, 214
  - interference
    - in NH_3 detn., 119
    - in Ca and Mg detn., 202, 204, 205, 207
    - in Cl detn., 74, 77, 78, 83
    - in ketone body detn., 109
    - in lactate detn., 107
    - in Mg detn., 64
    - in P detn., 97, 102
    - in K detn., 23
    - in serum conductivity measurements, 167, 169-171
    - in serum osmolarity detn. by boiling point method, 183
  - level
    - normal values, 4, 5
    - in renal in sufficiency, 300, 301
    - in uremia, 302
    - ultrafiltrable Ca and Mg detn. from, 66, 68-72
  - loss, in burn trauma, 396, 397, 399
  - Mg binding power, 58
  - -Mg complexes, in serum, 249
  - S level, detn. by X-ray spectroscopy, 215-217
- Blood serum
  - NH_3 detn. in, 119-123
    - normal values, 123
  - ashing for K detn. by chloroplatinate method, 32, 33

HCO_3-level
  in fever and hypothermia, 296-298
  relation to carbonic acid level pCO_2, 131-134
  in starvation, 295, 296, 298
  in uremia, 302
Br level in bromism, 403, 404
Ca detn. in
  atomic absorption spectrometry, 199-208
  AutoAnalyzer methods, 40, 41
  chelation methods, 37-40
  chemical methods, 37-55
  N-hydroxynaphthalimide method, 42, 43, 51-55
  nuclear fast red method, 37, 38, 44
  oxalate permanganate method, 48-51
  Trinder method, 51-55
  ultrafiltration method, 66-73
  X-ray fluorescence or emission spectroscopy methods, 43
Ca direct precipitation from, Pribrom discovery, 22
Ca level
  by atomic absorption spectrometry, 203, 205-207
  by calcein vs. Cal-red methods, 39
  by DCTA-Calcein method, 40
  by EDTA-Calcein method, 39
  by EDTA-Calcon method, 38
  by EDTA-Calmagite method, 40
  by EDTA-Cal-red method, 38, 39
  by flame photometry, 195
  in hyperatremia of children, 390
  reported values, 85, 159
  by X-ray spectroscopy, 226
  in starvation, 296
  Sulkowitch test and, 43
Ca and Mg detn
  detn. in by atomic absorption spectroscopy, 43
  direct titration in, 40
  polarographic, 43
pCO_2, detn.
  by electrometric method, 138-142
  by Henderson-Hasselbalch equation, 129-135
  by interpolation methods, 128
  value range by electrometric method, 141
carbonic acid level, relation to HCO_3- and pCO_2, 131-134
cation levels in
  reported values, 159
  detn. by flame photometry, 189-198
Cl detn. in
  argentimetric method, 81, 82
  colorimetric methods, 76
  iodometric methods, 75
  mercuric chloranilate method, 77-79
  mercurimetric method, 25, 75, 80, 81
  Volhard titration method, 74, 75
Cl level
  in familial periodic paralysis, 376
  in fever and hypothermia, 296-298
  in lobar pneumonia, 349-352
  normal values, 85
  in renal insufficiency, 300, 301
  in starvation, 294-296
  in uremia, 302
  by X-ray spectroscopy, 226
cholesterol level
  in renal insufficiency, 301
  in uremia, 302
chromogens, oxidation for Ca detn., 39
composition
  in lobar pneumonia, 350, 351
  in terminal uremia, 302
conductivity measurements
  protein interference in, 167, 169-171
  for total base and Na detn., 166-175
creatinine level in uremia, 302
deproteinization
  for NH_3 detn., 119
  for Ca and Mg detn., 202, 204, 205, 207
  for Cl detn., 74, 77, 78, 83
  for ketone body detn., 109
  for lactate detn., 107
  for Mg detn., 64
  for P detn., 97, 102
  for K detn., 23
  for SO_4 detn., 90-92
dilution problems in K detn., 21, 25
dilution with NaCl for Ca photometric detn., 41
dysproteinemic or icteric, Mg detn. in, 64
electrolyte levels in
  in normal values, 4, 5
  in values by X-ray spectroscopy, 225, 226
electrolyte patterns
  in acute and chronic renal insufficiency, 299-302
  in adrenocortical insufficiency, 300, 301
  in anorexia nervosa, 293-296, 298
  body temp. effects on, 293, 296-298
  in bone and parathyroid disease, 371, 372
  in Br intoxication, 403, 404
  in burn trauma, 394-401
  in cardiac decompensation, 300-301
  in diabetes mellitus, 362-367
  in disease, 293-298
  in fever and hypothermia, 293, 294, 296-298
  in glomerulonephritis, 299, 300
  in heart failure, 288
  in inanition, 293-296, 298
  in $HgCl_2$ poisoning, 300, 301
  in methanol intoxication, 404, 405
  in nephrosis, 300, 301
  in pneumonia, 293, 296-298
  in salicylate intoxication, 402, 403

  in terminal renal insufficiency, 301-305
  in uremia, 299, 300-305
electrolytes in
  detn. by conductivity methods, 166
  detn. by X-ray spectroscopy, 209-227
electrolyte studies on, historical aspects, 3
electrolyte and water levels in heart failure, 288-292
fatty acid detn. in by gas chromatography, 113-118
fatty acid levels in, clinical interpretation, 117
formic acid level in methanol intoxication, 105
heating and ashing for total base detn., 164
pH
  in acute acid-base disturbances, 265-272
  hyperventilation effects on, 341
icteric
  Ca detn. in, 39, 40
  Cl detn. in, 75
inorganic P and Mn direct precipitation from, discovery, 22, 23
alpha-ketoglutarate level in carbohydrate metabolism derangements, 106
ketone bodies in
  detn., 109-111
  value range, 112
lactic acid detn. in, Barker method, 107, 108
lactic acid level
  abnormal values, 104, 105
  in anoxia and shock, 105
lipemic, extration with ethyl ether for Ca detn., 39
Mg detn. in
  by atomic absorption spectrometry, 199-208
Bohoun method, 62-65
  by ultrafiltration, 66-73
Mg level
  abnormal values and related syndromes, 65
  and hypothermia, 58
  Mg intake as related, 250
  reported values, 58, 159, 322-325
  in starvation, 296
  in uremia, 302
  value range by atomic absorption spectrometry, 203, 205, 207
  value range by flame photometry, 197
methanol level in intoxication, 404
nonelectrolyte patterns in uremia, 301-305
organic acid detn. in, general aspects, 104-108
organic acid levels
  normal and abnormal values, 104, 105
  in renal failure, 106
  in renal insufficiency, 300, 301
  reported values, 159
osmolality detn.
  by freezing point method, Stadie-Sunderman modification, 177-181
  by freezing point method, survey of methods, 176, 177, 181
  indices, 288
  survey of methods, 181
  by vapor pressure osmometry, 181-188
osmotic pressure
  detn. and regulation, 6
  regulation, 273-275, 289
  in uremia, 300, 302, 305
  value range by vapor pressure osmometry, 187
oxalic acid level in hyperoxaluria, 106
perchloric acid filtrate, Mg photometric detn. in, 57
P detn. in
  automatic methods, 99
  general aspects, 97
  molybdate method, 101-103
P level
  glucagon effects on in familial periodic paralysis, 382
  normal values, 103
  in uremia, 302
  values by X-ray spectroscopy, 226
PO_4 level
  in renal insufficiency, 300, 301
  reported values, 159
K detn in
  chloroplatinate method, 31-33
  cobalti-nitrite method, 27-31
  tetraphenylboron method, 33-35
K level
  in cirrhosis, 356
  in familial periodic paralysis, 373, 380-384
  normal and abnormal values, 35
  in paramyotonia congenita, 373, 386, 387
  reported values, 29, 30, 159
  in uremia, 302
  value range by flame photometry, 197
  by platinic chloride vs. tetraphenylboron method, 35
  by X-ray spectroscopy, 226
preparatory treatment for gas liquid chromatography, 117
salicylate analysis in suspected intoxication, 105
sample prepn. for X-ray spectroscopy, 210-212, 223
Na detn. in
  chemical methods, 13
  colorimeter method, 15-18
  flame photometric method, 189-192
  gravimetric method, 8, 9, 13-15
  method survey, 8-18
  Sunderman method, 9, 13-15
  titrimetric method, 9-11
Na level
  in burn trauma, 398

    detn. by Sunderman conductimetric method, clinical aspects, 161
    factors conditioning, 338, 339
    in familial periodic paralysis, 376
    in heart failure, 288-292
    high values, pediatric aspects, 388, 389
    in liver disease, 354, 355
    normal values by conductivity method, 174
    normal values by flame photometry, 196
    as osmolarity index in heart failure, 288, 289
    reported values, 159
    in starvation, 295
    in uremia, 302
  SO_4 detn. in
    by chloranilate method, 92
    general aspects, 90-93
  SO_4 level
    clinical significance, 263
    normal values, 92, 96
    in renal insufficiency, 300, 301
    reported values, 159, 260
  S in, forms and values, 262
  S level, in multiple myeloma, 216, 217
  total base
    detn. by Sunderman conductimetric method, clinical aspects, 161
    Hald benzidine method for detn., 163-165
  total base values
    abnormal, 161, 162
    age differences in, 158, 159
    food intake effect on, 159
    in hypothermia, 296-298
    normal, 157-161, 165
    in renal insufficiency, 299, 300
    in starvation, 294-296
    by Sunderman conductivity method, 174
    in uremia, 302
    variability, 157-159
  total CO_2 detn. in
    gasometric method, 124-127
    value range, 127
  ultrafiltrable Ca in
    detn., 66-73
    normal values, 69
  ultrafiltrable Mg in
    detn., 66-73
    normal values, 69
  urea level in uremia, 302
  uric acid level in uremia, 302
  volume
    in febrile states, 296-298
    in starvation, 296-298
Blood sugar
  glucagon effects on in familial periodic paralysis, 382
  level in uremia, 302
  low, in salicylate intoxication, 403
  plasma osmolality calculations from, 341
Blood vessels, lesions, in Mg deficiency, 323
Blue diaper syndrome, renal parenchymal calcification in, 321
Body
  composition, variation with habitus and sex, 337, 338
  extremities, paralysis and hypokalemia, 29
  fat level, as criterion for parenteral fluid admn., 338
  position, ventilatory response to, 343, 344
  skin fold thickness, as criterion for parenteral fluid admn., 337, 338
  specific gravity, as criterion for parenteral fluid admn., 338
Body fluids
  balance
    in burn trauma, development of knowledge, 395
    in hypernatremia of children, 389, 390
    in infectious diseases of children, 392, 393
  colligative properties, 6
  compartmentalization, concept development, 3
  compartments
    pH gradients, 265-272
    model for cholera, 283
  distr.
    in burn trauma, 397, 398
    regulation, 6, 7
  pH, differentials in acute acid-base disturbances, 265-272
  interchange across capillary membranes, 7
  loss
    in Asiatic cholera, 280
    in diabetic acidosis, 362, 363
  methanol distr. in intoxication, 404
  osmotic equilibrium, maintenance, 5, 6, 273-275, 288, 289
  osmotic pressure
    detn., 6
    in heart failure, 288
  K detn. in, 29
    chemical methods, 19-35
  preparatory treatment for ketone body detn., 109
  replacement, in burn therapy, 394-400
  shifts
    in burn syndrome, 397-399
    in intestinal obstruction, 332-336
  Na detn. in
    methods, 8-18
    photometric methods, 11, 12
    titrimetric method, 9-11
  trans-capillary interchange, 7
Body temperature, blood pH dependence on, 147, 148
Body water
  balance, in lobar pneumonia, 351, 352

  level, as criterion for parenteral fluid admn., 338
  physicochemical state, 3, 4
Body weight
  as criterion for parenteral fluid admn., 337
  loss, in hypernatremia of children, 390
Bohoun method for Mg detn.,
  general considerations, 57, 62, 63
  principle, 63
  procedure, 64
  reagents, 63, 64
Boiling-point elevation, body fluid osmotic pressure detn. by, 6, 183
Boltz-Mellon absorption spectra curves for reduced molybdic acid complexes, 98
Boltz-Mellon method for P detn., 99
Bone
  brittleness, in Mg deficiency, 323
  Ca detn. in by chelation method, 40
  Ca and P components, 242
  crystal structure and hydration shell in fluid and electrolyte exchange, 368-370
  crystal surfaces, as Na reservoir in electrolyte disturbances, 390
  demineralization
    in diabetic acidosis, 365
    in hyperadrenocorticism, 244
    in hyperparathyroidism, 244
    in hyperthyroidism, 243
    in pregnancy, 243
    of renal origin, 245
  disease, electrolyte metabolism in, 368-372
  fibrous dysplasia, renal parenchymal calcification in, 321
  formation
    sex steroid effects on, 244
    somatotropin promoting effect on, 243
  ion polarity in, 369, 370
  mineral composition, 368
Bonneau, control analysis in K detn., 23, 24
Borax buffer
  standard
    composition, 152
    pH values and temp. correction, 151
Boric acid, soln., for NH_3 detn., 120
Boron, level in bone, 368
Böttger acidity vs. titratable acid detn. with H_2 gas electrode, 144
Bowser method for K detn., 21, 22
Bradbury method for Na detn., 11
Brain
  Br level in bromism, 403
  hypothalamus
    osmoreceptors in heart failure, 291
    osmotic activity control by, 274, 275
    solute excretion control by in hypernatremia of children, 389
  injury, hypernatremia in children and, 389, 390
  intracellular fluid, pH, blood and cerebrospinal fluid pH as related, 269
  lesions, in NaCl intoxication, 319
Brandstein-Castellano-Mezzacapa method for Ca, 41
Breh-Gaebler method for K detn., 23, 24
Briggs method for K detn., 23
Bromides
  interference in Cl detn., 84
  intoxication
    serum electrolyte patterns in, 403, 404
    therapy, 403
Bromine, detn. by X-ray spectroscopy, 219, 225
Bronchiectasis, anesthesia in, 342
Brush method for Ca detn., 39
Buffers, standard, for pH detn., 151-155
Bugarszky-Tangl conductivity measurements of serum, 167
Bunsen, flame spectra analysis, 189, 199
Burdick-Wildasin-Chang method for Ca detn., 38
Burns
  body fluid distr. in, 397
  Cl balance in, 394
  edema in, cause and characteristics, 396
  exudate loss in, 399
  fluid and electrolyte replacement therapy in, 395-399
  mortality due to, 395, 397
  occurrence frequency and prevention, 395
  protein leakage in, 396, 397
  renal glomerular permeability increase due to, 398
  serum electrolyte alterations due to, 394-401
  shock prevention in, 395, 397
  stress sensitivity in, 399
  therapy, historical account, 394, 395
  types, edema type as related, 396
Burn syndrome
  definition, 395, 396
  first phase, characteristics, 396-398
  second phase, characteristics, 398, 399
  third phase, characteristics, 399
  fourth phase, characteristics, 399, 400
Butler-Tuthill method for Na detn., 11
Butyric acid
  beta-hydroxy-
    accumulation in ketoacidosis, 105
    level in blood and urine, value range, 112
    oxidation to acetoacetic acid, 109-112
    standard soln. for ketone body detn., 110
Butterworth method for Na detn., 12

C

Calcein, Ca detn. with, 39, 40, 44
Calcein W, Ca detn. with, 39
Calcitonin, Ca metabolism response to, 245

Calcium
  acetylcholine release response to, 248
  detn., by atomic absorption spectrometry
    apparatus, 202
    evolution, 43, 199
    general considerations, 199-201
    instrument parameters, 203, 204
    interference phenomena, 204, 205, 207
    operating conditions, 203
    principle, 200, 201
    procedure, 202, 203
    reagents, 201, 202
    value range, 203, 205-207
  detn., by atomic absorption and X-ray emission spectroscopy, 45
  detn., by AutoAnalyzer, 38, 41, 42, 45
  detn., in biological materials, basic methods, 38
  detn., biological methods, 38
  detn., by chelation methods, 37-40, 44
  detn., in serum chemical method, 35-55
  detn., by flame photometry
    apparatus, 194
    error sources, 194, 195
    general aspects, 41, 42, 45
    principle, 193, 194
    procedure, 194
    reagents, 194
    standard solns., 194
    value range for serum, 195
  detn., fluorometric method, 39
  detn., by gasometric method, 38
  detn., by gravimetric methods, 38
  detn., Greenwald composite method for blood, 22
  detn., by N-hydroxynapthalimide method
    apparatus, 53
    calculations, 55
    general aspects, 42-45
    principle, 51
    reagents, 51-53
  detn., in icteric serum, 40
  detn., indicators for, 37-42, 44
  detn., miscellaneous methods, 43
  detn., by nephelonetry, 38
  detn., by nuclear fast red method, 37
  detn., by oxalate-permanganate method
    apparatus, 49
    calculations, 50
    principle, 48
    procedure, 49, 50
    reagents, 48, 49
  detn., by photometric methods, 38
  detn., by polarography, 38, 43
  detn., in presence of Mg, 38, 39
  detn., by spectrography, 38
    method, 39
    Trindor method, 51-55
  detn., in urine, Sulkowitch test and, 43
  detn., volumetric methods, 38
  detn., by X-ray fluorescence or emission spectroscopy, 43
  detn., by X-ray spectroscopy, 38, 223
  -C.I. Mordant Blue 13 complex, nature, 39
  direct precipitation from serum, Pribrom discovery, 22
  direct titration in serum, 40
  distr. in body, 242
  exchangeable, in bone, 370
  forms and functions in biological systems, 242
  imbalance
    in hypernatremia of children, 390
    renal lesions in, 321
  level in bone, 368
  level in serum
    reported values, 4, 5, 159
    in starvation, 296
    in uremia, 302
    value range by atomic absorption spectrometry, 203, 205-207
    by Calcein vs. Cal-red methods, 39
    by DCTA-Calcein method, 40
    EDTA-Calcein method, 39
    EDTA-Calcon method, 38, 39
    EDTA-Calmagite method, 40
    EDTA-Cal-red method, 38, 39
    EGTA-Calcon method, 38
    by flame photometry, 195
    X-ray spectroscopy, 226
  loss
    in diabetes, 364, 365
    in P deficiency, 322
  Mg detn. and, 64
  Mg separation from with ion exchange resin, 57, 58
  metabolism
    in anesthesia, 340
    control, general aspects, 242, 243
    endocrine regulation, 243-245
    gastrointestinal regulation, 245
    in lobar pneumonia, 351
    Mg intake effect on, 322-325
    nutritional factors in, 246
    in renal disease, 308, 309
    renal regulation, 245
  neuromuscular irritability response to, 242, 248
  precipitation as oxalate for flame photometric detn., 42
  protein-bound, detn., 43
  removal for Na detn., 8
  renal tubular precipitation, in Cl deficiency, 320
  spectral line in emission photometry, 193
  standard soln., prepn., 53, 194, 201, 211, 218
  total serum, ultrafiltrable Ca detn. from, 66, 69, 70

  - ultrafiltrable
    - normal values for serum, 69
    - detn. in serum, 66-70
  - ultrafiltrable, detn. in serum
    - apparatus, 67
    - calculations, 68-70
    - principle, 66
    - procedure, 68
- Calcium carbonate, Ca stock standard soln. prepn. with, 53
- Calcium fluoroapatite, in bone, 371
- Calcium gluconate, tetany treatment with in hypernatremia of children, 391
- Calcium hydroxide
  - powdered, in lactate detn., 107
  - urinary glucose removal with for ketone body detn., 109, 112
- Calcium hydroxide buffer, standard
  - composition, 152
  - pH values and temp. correction, 151
- Calcium hyroxyapatite, as bone primary structural element, 368
- Calcium oxalate
  - Ca detn. as, 48-51
  - perchloric acid-dissolved, flame photometry, 42
  - titration for manometric analysis, 43
- Calcium oxide, Ca detn. as, 38
- Calcium phosphate
  - Berzelius' analysis for muscle, 3
  - ubiquitousness in animal bodies, 3
- Calcium picrolinate, Ca detn. as, 38
- Calcium sulfate
  - excretion, values, 263
  - solubility factor in total base detn., 165
- Calcon, Ca detn. with, 38, 44
- Calmagite, Ca detn. with, 40, 44
- Cal-red, Ca detn. with, 38, 39, 44
- Cancer
  - colonic, serum total base values in, 161
  - gynecologic, serum lipid changes in, 117
- Capillaries
  - body fluid exchange, 7
  - properties, in burn syndrome first phase, 396
- Capillary tubes, in blood collection for pH electrometric detn., 150, 151
- Carbocel
  - Ca detn. with, 37
  - elimination of Mg interference in Ca detn., 37
- Carbohydrates
  - balance, in burn trauma, 399
  - gas chromatography, 113
  - interference in P detn., 97
  - metabolism
  - abnormality, in familial periodic paralysis, 373, 378-385
  - in paramyotonia congenita, 373, 385-387
    - K deficiency effects on, 366
- Carbonate
  - exchangeable, in bone, 370
  - level in bone, 368
- Carbon dioxide
  - compressed, in pCO$_2$ detn., 139
  - detn., by electrometric method
    - apparatus, 140
    - calculations, 141
    - general aspects, 138, 139
    - principle, 139
    - procedure, 140, 141
    - reagents, 139, 140
    - value range, 141
  - detn., by Henderson-Hasselbalch equation, 129-135
  - detn., by interpolation methods, 128
  - detn., by Natelson microgasometric method
    - apparatus, 125
    - calculations, 126
    - error sources, 127
    - principle, 124
    - procedure, 125, 126
    - reagents, 124, 125
  - detn. in serum, general aspects, 124
  - hydration to H$_2$CO$_3$ by carbonic anhydrase in acid-base disturbances, 267
  - inhalation, blood and cerebrospinal fluid pH changes in, 267
  - level in feces in Asiatic cholera, 280
  - metabolism, in salicylate intoxication, 402, 403
  - narcosis, respiratory acidosis in, 137
  - standard soln., prepn., 124, 125
  - tension
    - in alveolar gas vs. arterial blood, 341, 342
    - in anesthesia, 341-347
    - Cl shifts and, 254, 255, 257
    - clinical considerations, 135-137
    - role in blood and cerebrospinal fluid pH shifts, 265, 266, 267, 268, 269, 270
  - serum electrolyte response to, 341
    - serum ultrafiltration response to, 66
  - total
    - in blood, pCO$_2$ detn. from by interpolation, 128-134
    - in blood, clinical considerations, 135-137
    - in serum, value range, 127
- Carbonic acid
  - formation and dissociation in Cl shift, 254, 255
  - level in serum and relation to HCO$_3^-$ and pCO$_2$ partial pressure, 131-134
  - transfer among body fluid compartments in acid-base disturbances, 267
- Carbonic anhydrase
  - activity in kidney in K depletion, 315
  - HCO$_3$— dehydration in Cl shift, 255
  - CO$_2$ hydration by in Cl shift, 254, 267
  - inhibitors, diuretic action in K depletion, 265

- Carbon tetrachloride, intoxication, hepatic and renal necrosis in, 353
- Carboxanilide dye method for Mg, Bohoun modification, 57, 62-65
- Carboxylase, Mg activation, 248
- Carcinoma
  - bronchogenic, electrolyte abnormalities in, 277, 278
  - prostatic, with terminal uremia, serum composition in, 302
- Carius method for deproteinization, 74
- Carr-Frank method for Mg detn., 57
- Cations
  - active transport, Na role in, 239, 240
  - cellular extrusion, Cl transport as related, 254-257
  - detn. by flame photometry, 189-198
  - level in cell, relation to active transport, 238-240
  - level in feces vs. plasma in diarrhea, 283-286
  - level in serum, reported values, 4, 5, 159
  - loss due to emesis in uremia, 300
- Celite 545, coated column, serum fatty acid chromatography on, 114
- Cells
  - electrochemical gradient with extracellular fluid, Cl shifts in, 254, 255
  - pH shifts in acid-base disturbances, 265-272
  - Mg component, serum Mg detn. as complicated by, 63
  - osmotic equilibration with external fluids, 5, 6
  - K level, normal values, 20
  - respiration, ADP control of, 240
- Centrifuge tubes
  - for Ca detn.
    - by Clark-Collip method, 49
    - by N-hydroxynaphthalimide method, 53
- Cerebrospinal fluid
  - Br level in bromism, 403
  - Ca detn. in, nuclear fast red method, 37
  - pH, in acute acid-base disturbances, 265-272
  - methanol level in intoxication, 404
  - proteins, hypernatremia of children and, 389
  - tap site for acid-base balance studies, 266, 268
- Cesium, K displacement by, acid-base balance response to, 265
- Chelating agents, Ca detn. with, 37, 42, 44, 45
- Children
  - electrolyte metabolism disturbances in, 388-393
  - fluid and electrolyte balance in infectious diseases, 392, 393
  - hypernatremia, clinical picture and therapy, 388-391
  - metabolic alkalosis
    - clinical picture and therapy, 391, 392
    - due to persistent emesis, 391, 392
  - seizures, in hypernatremia, barbiturates and paraldehyde in control of, 391
  - serum K level, 29, 30
- Chloride
  - balance
    - in burn trauma, early knowledge, 394
    - disturbances, 257, 258
    - maintenance, 253, 254
    - in metabolic alkalosis of children, 391, 392
    - in pregnancy and lactation, 255
  - Berzelius' analysis for muscle, 3
  - Br replacement of, 403
  - bromism treatment with, 404
  - Ca flame photometric detn. in presence of, 42
  - deficiency, Ca metabolism in, 320
  - detn.
    - absorption indicator methods, 75
    - argentimetric method, 81, 83
    - colorimetric methods, 76-89
    - electrometric methods, 75-77
    - in icteric serum, 75
    - interference phenomena, 83, 84
    - iodometric methods, 75
    - methodological discussion, 83,-86
    - microdiffusion method, 75
    - Volhard titration method, 74-76
    - by X-ray spectroscopy, 223
  - detn. by mercuric chloranilate method
    - evaluation, 83, 84
    - principle, 77
    - reagents, 77-78
  - detn. by mercurimetric method
    - calculation, 81
    - evaluation, 84, 85
    - general survey, 75
    - principle, 80
    - procedure, 81
    - reagents, 80, 81
  - distr. and exchange in bone, 370
  - distr. and transport, 254-256
  - excess, renal lesions due to, 319-321
  - excretion, in familial periodic paralysis, 376, 381
  - gastric transport, 255, 256
  - imbalance, renal lesions in, 319-321
  - intestinal transport, 256
  - level in body fluids, normal values, 85
  - level in bone, 368
  - level in edema fluid in burn trauma, 397
  - level in feces in Asiatic cholera, 280
  - level in serum
    - in familial periodic paralysis, 376
    - in fever and hypothermia, 296-298
    - in renal insufficiency, 300, 301
    - reported values, 159
    - in starvation, 294-296
    - in uremia, 302
  - loss
    - in diabetes, 363
    - due to emesis in uremia, 300
    - in intestinal obstruction, 333
    - in $HgCl_2$ poisoning, 301

    routes and value range, 253, 254
  metabolism
    abnormalities, in tuberculosis, 278
    in anesthesia, 341
    in infection, 349-352
    pitressin effects on, 277
    in pneumonia and other infectious diseases, 349-352
    regulation, 253-259
    in renal disease, 308
  renal transport, 256-257
  requirements, value range, 253
  retention
    in burn trauma, 396
    in lipoid nephrosis, 301, 302
  serum total base in, 158, 159
  shift, as error source in total base detn., 165
  standard soln. for X-ray spectroscopy, 211
  transport
    anion shifts and, 254
    in intestinal obstruction, 333
  ubiquitousness in animal bodies, 3
Chloride space, as extracellular fluid vol. index, 254
Chlorine
  level in serum, values by X-ray spectrometry, 226
  standard soln. for X-ray spectroscopy, 218
Chloroplatinate method for K
  apparatus and reagents, 32
  calculations, 33
  general aspects, 20, 21
  as preparatory method for Na detn., 8, 9, 25
  principle and reactions, 31
  procedure, 32, 33
  range of values, 30, 35
  reagent, 32
  unsatisfactory aspects, 20, 22
Chlorthiazide, vacuolar nephropathy due to, 312
Cholangiitis, vacuolar nephropathy in, 312
Chlorea
  fecal vs. plasma alkali metal levels in, 282
  fluid compartment model for, 283
  fluid and electrolyte disturbances in, 280
  vacuolar nephropathy in, 312
Cholesterol
  level in serum
    P level as related, 226
    in renal insufficiency, 301
    in uremia, 302
Christiansen ionometer for serum conductivity measurements, 167-169
Chromatographs, gas, manufacturers, 113
Chromatography
  gas
    branched-chain fatty acid identification by, 117
    clinicochemical applications, 113
    of Kreb's cycle intermediates, 117
    Martin-Synge innovation, 113
    serum fatty acid detn. by, evaluation, 116
    serum fatty acid detn. by, principle, 113, 114
    serum fatty acid detn. by, procedure, 115, 116
    serum fatty acid detn. by, reagents, 114
    serum fatty acid detn. by, standard solns., 114
  gas liquid
    amino acid separation by, 117
    serum fatty acid detn. by, 113-118
  silicic acid column, preparatory for fatty acid chromatography, 115
  thin layer, preparatory for fatty acid gas chromatography, 115
Chromium, detn.
  by flame photometry, 42
  by X-ray spectroscopy, 218, 219
Chromium sesquioxide, as fecal reference in Ca balance studies, 42
Circulation
  embarassment, in interstinal obstruction, 333, 335
  Mg excess effects on, 250
Cirrhosis
  advanced, iodocyanine green clearance in, 339, 360
  alcoholic
    plasma vol. in, paracentesis effect on, 357
    K imbalance in, 356
    reversible azotemia in, 358
    Na and water imbalance in, 354, 355
    water and electrolyte imbalance in, 354-356
  ascitic, in aldosteronism, 230
  ion transport mechanism impairment in, 241
  lowered serum Mg in, 250
  serum total base values in, 161
  with terminal uremia, serum composition in, 302
  water and electrolyte imbalance in, 353, 354
Citrate
  Ca detn., in presence of, 41
  distr., form and level in bone, 370, 371
Citric acid
  secretion, in K depletion,
  level in serum, diagnostic significance, 104
Clark-Collip method for Ca detn.
  as reference method, 48-51
  vs. other methods, 37, 39, 42, 43, 44
Clark method for Na detn., 10
Clark method for Cl detn., 75
Clausen method for K detn., 22
Clostridium, toxin, role in intestinal obstruction, 335
Cobalt
  detn. by X-ray spectroscopy, 218, 219
  level in bone, 370

  precipitation in cobalti-nitrite reaction for K, 21, 22
Cobalti-nitrite method for K
  De Koninck as innovator, 20
  general considerations, 19-26
  principle and reactions, 27
  procedure, 28-31
  range of values, 30
  reagents for, 27, 28
Cobalti-nitrite reagent
  ion specificity in precipitation reactions, 20
  stepwise addition for more complete K precipitation, 21
Cold, myotonia accentuation by, 385, 386
Coliform bacilli, toxins, role in intestinal obstruction, 335
Colitis, ulcerative
  K depletion due to, 312
  vacuolar nephropathy in, 310, 311
Collagen, Cl binding, 254
Colon, cancer, serum total base values in, 161
Colorimetry
  lactate detn. by, 107, 108
  in P detn., 102, 103
  in K detn., 23, 24
  in Na detn., 11, 12
C.I. Mordant Black 11
  Ca detn. with, 38, 40
  Mg detn. with, 57
C.I. Mordant Blue 13
  Ca detn. with, 39, 40, 44
  Ca complex, nature, 39
  Mg detn. with, 57
Coma
  amoniagenic, clinical laboratory aspects, 119
  diabetic, hypermagnesemia in, 250
Complexometric method, for Na detn., 10, 11
Conductivity method
  for Na, 9
  for total base and Na detn., 166-175
Conn's syndrome, *See* Aldosteronism
Convulsions
  in Mg deficiency, 323
  in metabolic alkalosis of children, 391
Conway method for Cl detn., 75
Coolidge method for Ca detn., 40
Copeland-Sunderman equation for ultrafiltrable Ca, 66, 69
Cope method for sulfate detn., 91
Copper
  Ca and P metabolism response to, 246
  detn. by X-ray spectroscopy, 218
  electroplating for X-ray spectroscopy, 214
  level in bone, 368
Copper sulfate
  soln., for lactate detn., prepn., 107
  urinary glucose removal with for ketone body detn., 109, 112
Copp method for Ca detn., 37
Corinth Ca, Ca detn. with, 41
Corn oil, K-deficiency symptoms intensification by, 317
Corticosteroids, glucogenic, carbohydrate metabolism response to familial periodic paralysis, 380, 383
Corticosterone, deoxy-, Cl metabolism response to in lobar pneumonia, 350
Cotlove electrometric Cl detn. method, 75, 76
Counter-current distribution, preparatory, for fatty acid gas chromatography, 115
Creatinine, level in serum in uremia, 302
Cresolphthalein, Ca and P detn. with, 41
Cuthbertson method for SO_4 detn., 91
Cyclopentane-5-carboxylic acid, 1-amino-, Na-dependent transport, 233, 236, 238
Cysteine, S utilization from, 260
Cystinosis, K depletion in, 312
Cystitis, in NaCl intoxication, 319

D

Danio, SO_4 absorption from sea water, 261
DCTA, *See* Acetic acid, (1,2-cyclohexylenedinitrilo) tetra-.
DeBoer method for sulfate in blood, 90
Dehydration
    aldosteronism in, 230
  in Asiatic cholera, 280
  in diabetic acidosis, 363
  hyperkalemia in, 29, 35
  hypermagnesemia in, 250
  hypernatremia and hyperchloridemia in, 319
  hypertonic
    clinical picture in children, 389
    synonymy with hypernatremia, 388
De Koninck method for K detn., 20
Del Huega method for Ca detn., 42
Delirium tremens, lowered serum Mg in, 250
Denis method for SO_4 in blood, 90
Densitometer, for iodocyanine green clearance studies, 360
Deproteinizing reagent
  for Cl detn., 77
  for ketone body detn., 110
Dextrane, infusion, burn therapy with, 398
Dextrose
  infusion, in burn therapy, 397, 399
  soln., hypertonic, water metabolism response to, 275
Diabetes
  insipidus
    acquired vs. hereditary types, 276
    characterization, 276
  ketotic, serum keto acid levels in, 104
  mellitus, electrolyte metabolism in, 362-367
  Pheniform-treated, lactic acidosis in, 105

  - of pregnancy, aldosteronism in, 230
  - serum organic acids in, 104, 105
  - with terminal uremia, serum composition in, 302
- Dialysis, artificial, in salicylate intoxication therapy, 403
- Diaphorase, activity in kidney in K depletion, 315
- Diaphragm
  - immobilation, ventilatory response to in anesthesia, 343
  - Na-dependent non-electrolyte transport, 233
  - ventilatory efficiency in anesthesia, body position effects on, 344
- Diarrhea
  - bloody, in Mg deficiency, 323
  - Cl and K loss in, 257
  - fluid and electrolyte disturbances in, 280-287
  - fluid and electrolyte loss due to infections, 392
  - hypernatremia due to, pediatric aspects, 388
  - K loss due to, 29, 35, 311, 312
  - Na loss in, 307
  - vacuolar nephropathy in, 310, 311
- Dichromate
  - beta-hydroxybutyrate oxidation with, 109, 110
  - Na detn. with, 11
- Diethylene glycol, succinate polyester, Celite column coated with for fatty acid detn., 114
- Dimethylformamide, Na detn. with, 12
- Dioxane, poisoning, vacuolar nephropathy in, 310
- Diphenylcarbazone, Cl detn. with, innovation and modifications, 75, 81
- Diphosphopyridine nucleotide diaphorase, activity in kidney in Mg deficiency, 324
- Dipotassium sodium cobalti-nitrite
  - $KMnO_4$ reaction with, 21
  - K precipitation from, 22, 23
  - precipitate, constitution and solubility, 21
  - precipitation reactions, 20, 21
- Disease
  - infectious
    - of children, fluid and electrolyte balance in, 392, 393
    - serum electrolytes in, 349-352
  - inflammatory, intestinal obstruction due to, 332
  - serum electrolyte patterns to, 293-298
  - serum total base values in, 161
- Distillation apparatus, for sample prepn. in X-ray spectroscopy, 213
- Distillation methods for acetone, 109
- Diuresis
  - in burn trauma, 398, 399
  - glycosuric, in diabetes mellitus, 362
  - osmotic, in diabetes, 362
  - K loss due to, 29, 35
  - water, Na balance and, 307
- Diuretics
  - Cl balance response to, 258
  - electrolyte loss due to in cirrhosis, 355, 356
  - in liver disease, 359
  - osmotic, in heart failure, 291
  - renal response to in acid-base disturbances, 265
- Dressings, pressure, in burn therapy, 394
- Drugs
  - anesthesia depth response to, 341, 342
  - distr. in acid-base disturbance, 270
  - osmoregulatory system response to, 275
- Drushel method for K detn., 20, 21
- Dubsky-Trtilek method for Cl detn., 75
- Dyer method for P detn., 99
- Dysentery, bacillary, vacuolar nephropathy in, 312

E

- Ebden-Myerhoff pathway, in familial periodic paralysis, 383
- Eclampsia, lowered serum Mg in, 250
- Edema
  - in aldosteronism, 229, 230
  - cause and characteristics in burn syndrome first phase, 396
  - fluid, composition in burn trauma, 397
  - protein-rich loss in burn trauma, 396, 399
  - in heart failure, mechanism, 288-292
  - massive pulmonary, due to burn trauma, 398
  - pulmonary, respiratory acidosis in, 137
  - in rehydration for hypernatremia of children, 391
  - renal, serum total base values in, 161
  - in renal insufficiency, 300, 301
- EDTA, *See* Acetic acid, (ethylenedinitrilo)-tetra-.
- Effort syndrome, respiratory alkalosis in, 137
- EGTA, *See* Acetic acid, [ethylenebis (oxyethylenenitrilo)] tetra-.
- Ehrlich ascites tumor, Na-dependent non-electrolyte transport, 233
- Eisenman's interpolation method for pCO_2 logarithmic plot, 128, 129
- Electrocardiogram
  - in metabolic alkalosis of children, 392
  - in selective aldosteronism, 230
- Electrode cell assembly
  - for pH detn.
    - cell junction significance, 146
    - operational aspects, 144
    - temp. equilibrium in, 146-148
  - for pH electrode and buffer standardization, 146
  - potentials, pH meter in detn. of, 148-150
- Electrode potentials
  - quantitation by pH meter, 148-150
  - of standard hydrogen electrode, 144
- Electrodes
  - antimony-antimony oxide, Bates evaluation, 145
  - calomel
    - cleaning, 155
    - thermal stability, 146

    calomel reference, for pH detn., 147
    for pCO_2 detn., 138, 139
    decoating soln., for Sunderman conductivity apparatus, 172, 173
    glass
        Bates evaluation, 145, 153
        cleaning and storage, 152-154
        commercial, characteristics, 153
        for pH detn., 145-147
        surface injuries and potential deviations, 146, 153, 154
        thermal stability, 146
    pH
        electrical phenomena, 148, 149
        principle, 143
    quinhydrone, Bates evaluation, 145
    reference, principle, 143
    reversible hydrogen ion sensitive, principles, 143
    silver-silver chloride, as reference electrode, 145
    standard hydrogen, principles, 143-145
Electroencephalogram, abnormal, in children with hypernatremia, 389
Electrolytes
    balance
        in burn trauma, 394, 395, 399
        in infectious diseases of children, 392, 393
        maintenance, 5, 6
    in biological fluids, general aspects, 3-7
    detn.,
        by conductivity methods, 166-175
        Greenwald composite method for blood, 22
        by X-ray spectroscopy, 209-226
    detn. by X-ray spectroscopy
        apparatus, 220-223
        calculations, 224
        clinical interpretations, 226
        error sources, 225
        general aspects, 209, 210
        principle, 211-218
        procedure, 223, 224
        reagents, 218-220
        value range, 225, 226
    distr.,
        in burn trauma, 396, 398
        regulation, 6, 7
    electroplating for X-ray spectroscopy, 214
    exchange between blood and interstices, 7
    imbalance
        bone crystal surfaces as Na reservoir in, 390
        in cirrhosis, 354-356
        in liver disease, 353-361
    levels
        in body fluids, normal values, 4, 5
        in serum, values by X-ray spectroscopy, 225, 226
    loss in diabetes acidosis, 362-366
    loss and replenishment, 293, 298
    metabolism
        and narcosis, 337-348
        in anesthesia, 337-348
        in antidiuretic hormone deficiency, 276
        in bone and parathyroid disease, 368-372
        in bronchogenic carcinoma, 277
        in CNS disease, 277
        in diabetes mellitus, 362-367
        disturbances, pediatric aspects, 388-393
        in heart failure, 288-292
        in intestinal obstruction, 332
        notation units, 4
        in osteoporosis, 371
        in periodic paralyses, 373-387
        pitressin effects on, 277
        regulation in heart failure, 288-292
        in renal disease, 306-309
        in tuberculosis, 278
    in muscle and serum, historical aspects, 3
    osmolality, 6
    patterns
        in acute and chronic renal insufficiency, 299-302
        in adrenocortical insufficiency, 300, 301
        in anorexia nervosa, 293-296, 298
        body temp. effects on, 293, 296-298
        in Br intoxication, 403, 404
        in burn trauma, 394-401
        in cardiac decompensation, 300, 301
        in disease. 293-298
        in fever and hypothermia, 293, 294, 296-298
        in glomerulonephritis, 299, 300
        in inanition, 293, 294, 296-298
        in infectious diseases, 350
        in $HgCl_2$ poisoning, 300, 301
        in methanol intoxication, 404, 405
        in nephrosis, 300, 301
        in pneumonia, 293, 296-298
        in renal disease, 299-331
        in salicylate intoxication, 402, 403
        in terminal renal insufficiency, 301-305
        in uremia, 299-305
    replacement, in burn therapy, 394-400
    shifts, in burn syndrome, 397, 398
Electrometric method for Cl detn.
    critique, 76, 77
    survey, 75, 76
Electrometric method for Na, 9
Electromotive force, -pH relationships, 143, 144
Electron probe, X-ray spectroscopy with, 210, 218, 219
Electroplating instrument, in sample prepn. for X-ray spectroscopy, 214
Elements, transition, detn. by X-ray spectroscopy, 218
Elon, *See* Phenol, p-aminomethyl-, sulfate.
Emesis
    cation loss due to during starvation, 295, 298

  - Cl loss due to, 257, 300
  - fluid and electrolyte loss due to in infections, 392
  - in hypernatremia of children, 388, 389
  - metabolic alkalosis due to in children, 391, 392
  - K loss due to, 29, 35, 312
  - Na loss due to, 307
  - serum total base loss due to, 162
- Emphysema
  - anesthesia in, 342
  - obstructive, respiratory acidosis in, 137
- Encephalitis, respiratory alkalosis in, 137
- Encephalopathy, in hepatic disease, correction, 359
- Endocrine system
  - Ca metabolism control, 243-245
  - Cl transport regulation, 255, 256
  - milk composition regulation, 255
  - P metabolism control, 243-245
  - SO_4 metabolism regulation by, 262
  - sweat composition regulation, 255
- Enolase, Mg activation, 248
- Enteritis, regional, vacuolar nephropathy in, 312
- Enzymes
  - activity
    - in familial periodic paralysis, 379, 380, 383
    - in kidney in hypokalemia, 315, 317
  - inhibition, anesthesia depth response to, 341
  - interference in serum NH_3 detn., 119
  - Mg-specific, 248
- Eosin, Cl detn. with, 75
- Eosinophils, count, high, in familial perdiodic paralysis, 373
- Epilepsy, lowered serum Mg in, 250
- Epinephrine
  - carbohydrate metabolism response to in familial periodic paralysis, 380, 382, 383
  - hypokalemia due to, 29
  - paretic episode precipitation by in familial periodic paresis, 378
  - renal function response to, 229, 231
  - skin active Cl transport stimulation by, 254
- Eriochrome Black T., *See* C.I. Mordant Black 11.
- Eriochrome Blue SE, *See* C.I. Mordant Blue 13.
- Erio SE, *See* C.I. Mordant Blue 13.
- Erythrocytes
  - Cl shift, mechanism, 254, 255
  - count, in high intestinal obstruction, 333
  - Mg level in deficiency states, 324
  - level, 35
  - S in, forms, 262
  - Zn level in, values by X-ray spectroscopy, 226
- *Escherichia coli*, diarrhea due to, mechanism, 280
- Esterase
  - activity, in kidney in Mg deficiency, 324
  - activity, in kidney in K depletion, 315
- Estrogens
  - Ca and P metabolism response to, 244
  - S conjugation with, 262
- Ethanol
  - -ammonium hydroxide wash soln. for Mg detn., prepn., 61
  - benzidine reagent prepn. with, 94
  - in cobalti-nitrite reaction for K, 29
  - interference in ketone body detn., 109
  - intoxication, acid-base equilibrium in, 106
  - methanol oxidation inhibition by, 404
  - neurohypophyseal system response to, 275
  - in platinic chloride method for serum K, 32, 33
  - potassium cobalti-nitrite precipitate solubility in, 20, 25, 27
  - soln., for ketone body detn., 110
- Ethanolamine, N-hydroxynaphthal-imide Ca reagent prepn. with, 51
- Ethylene glycol, poisoning vacuolar nephropathy in, 310
- Ethyl ether
  - benzidine reagent prepn. with, 94
  - lipemic serum extraction with for Ca detn., 39
- Exercise, prophylactic effect in familial periodic paralysis, 373, 379, 383
- Extracellular fluid
  - body per cent composition by weight, 3
  - Ca and P components, 242
  - electrochemical gradient with cells, Cl shifts in, 254, 255
  - electrolyte levels in, normal values, 4, 5
  - osmolality, in heart failure, 288, 289
  - K level and regulation, 20
  - volume
    - Cl space and, 254
    - in febrile states, 296-298
    - in starvation, 296-298
- Exudates, fatty acids in, gas chromatography, 117
- Eye, retina, methanol toxic effects on, 404
- Eyelid, lag phenomenon in paramyotonia congenita, 386

F

- Face, muscles, myotonia in paramyotonia congenita, 385, 386
- Fajans-Hassel method for Cl, 75
- Fanconi syndrome
  - K depletion in, 312
  - renal parenchymal calcification in, 321
- Fantus method for Cl in urine, 76
- Fat
  - balance, in burn trauma, 399
  - Ca and P metabolism response to, 246
  - interference in K detn., 23
- Fatty acids
  - branched-chain, detn. in blood by chromatography, 117
  - in blood, exudates, and transudates, 117
  - detn. in serum by gas chromatography appartus, 114, 115

    - error sources, 116, 117
    - general aspects, 113
    - principle, 113, 114
    - procedure, 115, 116
    - reagents, 114
    - standard solns., prepn., 114
  - level in serum, clinical interpretation, 117
  - methyl esters, reference standards, 114
  - volatile, initial separation by gas chromatography, 113
- Fawcett-Wynn method for Ca detn., 41
- Fawcett-Wynn method for Mg detn., 57
- Fear, osmoregulatory system response to, 275
- Feces
  - alkali metals in, level in cholera, 282
  - Ca and Cr photometric detn. in, 42
  - cation levels in diarrhea, 283-286
  - Cl loss in, 253
  - composition, in Asiatic cholera, 280
  - Na transport inhibitor in diarrhea, 280, 281
- Feigl studies on molybdic acid reduction, 97
- Ferric ammonium sulfate, indicator for Cl detn., 82
- Ferric chloride, SO$_4$ detn. with by benzidine method, 91
- Ferric nitrate
  - acid soln. for Ca detn., 42
  - -nitric acid mixture for Ca detn., prepn., 52, 53
- Ferric perchlorate, Cl detn. with, 76
- Ferric sulfate, soln., total base detn. with, 163, 164
- Ferrocyanide, K detn. with, 24
- Fever
  - body fluid vol. in, 296-298
  - Cl metabolism as related, 350
  - fluid and electrolyte loss due to in infectious disease, 392
  - N balance in, 297
  - in salicylate poisoning, 403
  - serum electrolyte patterns in, 293
- Filter paper, sample collection on and positioning for X-ray spectroscopy, 210-215, 220, 223
- Fingerhut-Marsh method for bile pigment destruction in icteric serum, 75
- Fingerhut-Miller method for Ca detn., 39
- Fiske-Litarczek method for K detn., 24
- Fiske method for sulfate, 90
- Fiske method for urinary cations, Stadie-Ross adaptation to serum total base, 157
- Fiske-Subbarow method for P detn., 41, 98
- Fistula, K loss due to, 29
- Fluorescein, Cl detn. with, 75
- Fluorescence, X-ray, Ca detn. by, 43
- Fluoride
  - Ca and P metabolism response to, 246
  - distr. and form in bone, 371
  - level in bone, 368
- Fluorometry, Ca detn. by, 39
- Folic acid, depletion, liver regeneration in, 360
- Folin-Ciocalteau, reagent, in cobalti-nitrite reaction for K., 27, 29
- Food
  - intake
    - serum K and, 35
    - serum total base response to, 159
- Formaldehyde
  - deleterious effects in methanol intoxication, 404
  - soln., for tetraphenylboron method for K, 34
- Formic acid
  - deleterious effects in methanol intoxication, 404
  - level in serum
    - diagnostic significance, 104
    - in methanol intoxication, 105
- Freezing, serum NH$_3$ stabilization by, 119
- Freezing-point depression, in osmotic pressure detn., 6, 183, 288
- Fresenius, observations on K precipitate, 20
- Frog heart, Ca detn. with, 38
- Frog skin, Na transport inhibitor assessment by, 281

G

- Galactose, Na-dependent transport, 233
- Gambino method for blood collection in pH detn., 150, 151
- Gastritis
  - hemorrhagic, in salicylate poisoning, 403
  - lowered serum Mg in, 250
  - in NaCl intoxication, 319
- Gastrointestinal system
  - Ca metabolism control, 245
  - Ca and P absorption from
    - cortical steroid effects on, 244
    - in hyperthyroidism, 243, 244
    - parathhormone effects on, 244
    - sex steroid effects on, 244
  - Cl absorption in lobar pneumonia, 350
  - Cl conservation, 253
  - Cl loss from, 253
  - disease, vacuolar nephropathy in, 311
  - fluid reabsorption failure in diarrhea, 280
  - P metabolism control, 245
  - suction, anesthesia depth response to, 341
  - water and electrolyte fluxes in cholera, 283
- Gauvin benzidine method for urinary sulfate, 90
- Geiss reaction, in K detn., 23
- Gerlach-Frazier method for Cl detn., 75
- Germanium, molybdic acid complex, absorption spectrum, 98
- Gingiva, connective tissue, hypertrophy in Mg deficiency, 323
- Globulin
  - base-binding capacity, 160
  - Mg binding power, 58
- Glomerulonephritis

  - chronic, serum electrolyte patterns in, 299, 300
  - renal parenchymal calcification in, 321
- Glottis, necrosis, in NaCl intoxication, 319
- Glucagon, carbohydrate metabolism response to in familial periodic paralysis, 380, 382, 383
- Glucitol, 1-5-anhydro-, Na-dependent transport, 235
- Gluconates, interference in Mg detn. by titan yellow, 57
- Glucose
  - and analogues, Na-dependent transport, 233
  - hypokalemia due to, 29, 35
  - metabolism
    - in familial periodic paralysis, 378-380, 383
    - P role in, 366
  - removal from urine for ketone body detn., 109, 112
  - requirements for intestinal water transport, 334
  - solns., osmolality, 6, 288
- Glucose-1-phosphate, as glycogen metabolism intermediate in familial periodic paralysis, 379, 380
- Glucose 6-phosphate dehydrogenase, activity in kidney in Na and Cl imbalance, 321
- Glutamic acid, Na-dependent transport, 233
- Glutaminase, activity in kidney in K depletion, 315
- Glutaric acid
  - alpha-oxo-, accumulation in serum in liver disorders, 106
  - alpha-keto-, level in serum, diagnostic significance, 104
- Glutathione, S incorporation, 261
- Glutathione, as S source, 260
- Glycine
  - potassium sodium cobalti-nitrite reactions in presence of, 27
  - Na-dependent transport, 233
  - soln. for K detn. by cobalti-nitrite method, 28
- Glycogen, formation and breakdown in familial periodic paralysis, 379, 380, 382, 383
- Glycogenolysis, hepatic, anesthesia depth response to, 341
- Glycoprotein, S utilization from, 260
- Glycosides, cardiotonic, Cl imbalance due to, 258
- Glycosuria, diabetic, electrolyte and water loss due to, 362
- Golby-Hildebrand-Reilley method for Ca detn., modification, 38
- Gold
  - detn. by X-ray spectroscopy, 217, 218
  - electroplating for X-ray spectroscopy, 214
- Gomori method for P detn., 98
- Gonads, Ca and P metabolism control by, 244
- Gram-Cullen equation for corrected serum conductivity values, 169
- Gran method for Ca detn., 42
- Grass staggers, in Mg deficience, 249
- Greenwald method for electrolytes, 22
- Gum ghatti
  - color stabilization of titan yellow reaction for Mg, 57
  - soln., for tetraphenylboron method for K, 34

H

- Haessler, inorganic P and Mn direct precipitation from serum, 22, 23
- Haldane's solution, infusion, in burn trauma therapy, 398
- Hald benzidine method for total base detn.
  - apparatus, 163, 164
  - error sources, 165
  - principle, 157, 163
  - procedure, 164
  - reactions, 158
  - reagents, 163
- Hamburger method for K detn., 22
- Hano method for Cl detn., 76
- Hausman method for Cl detn., 76
- Headache, in metabolic alkalosis of children, 391
- Heart
  - arrhythmia, in familial periodic paralysis, 373
  - Ca effects on, assay aspects, 38
  - decompensation, serum electrolyte patterns in, 300, 301
  - failure
    - congestive, lowered serum Mg in, 250
    - electrolyte metabolism in, 288-292
    - electrolyte and water metabolism in, 288-292
    - ion transport mechanism impairment in, 241
    - K loss in, 289
    - Na loss in, 288-292
    - with terminal uremia, serum composition in, 302, 303
  - function, Mg excess effects on, 250
  - lesions, in Mg deficiency, 323
  - output, anesthesia depth response to, 341
- Helium, as carrier gas for fatty acid chromatography, 114
- Hematocrit
  - in burn therapy management, 399
  - level, body habitus and sex as related, 337, 338
- Hemoglobin, level, in high intestinal obstruction, 333
- Hemolysis
  - interference
    - in serum total base detn., 165
    - in SO_4 detn., 260
  - P false values due to, 103
- Hemorrhage, anesthesia depth response to, 341
- Henderson-Hasselbalch equation
  - for acid-base balance relationships, 267
  - for serum pCO_2 detn.
    - factor tables, 131, 132

    general aspects, 128
    graphical and mechanical soln., 129-134
    nomograms, 132-134
    slide rule soln. of, 133
  McLean nomogram for soln. of, 132
  Van-Slyke-Sendroy nomogram in soln. of, 132
Henry method for Na detn., 15-18
Heparin, interference in P detn., 103
Hepatitis
  serum, as complication of burn trauma, 399
  serum total base values in, 161, 162
Hernia, external, intestinal obstruction due to, 332
Heteropoly blue, the term, origin and definition, 99
Hexokinase, Mg activation, 248
Hexose phosphate pathway, in familial periodic paralysis, 379
Hippuric acid, p-amino-, renal transport in K depletion, 318
Histamine, release in Mg deficiency, 323
Hoffman method for Na detn., 11
Hoffman method for SO_4 detn., 91
Hormones, osmoregulatory system response to, 275
Hubbard method for SO_4 detn., 91
Hyaline droplets, renal
  degeneration in hypokalemia, 311, 313
  in hypernatremia and hyperchloridemia, 319
  in hypokalemia, properties, 310, 311, 313, 315
Hydrazine, (2,4-dinitrophenyl)-, acetone detn. with, 109
Hydrazine sulfate, phosphomolybdate reduction with, 99
Hydriodic acid, phosphomolybdate reduction with, 99
Hydrocephalus, vascular nephropathy in, 312
Hydrochloric acid
  benzidine reagent prepn. with, 94
  body fluid compartment pH shifts due to infusion of, 265-270
  Ca detn. with, 42, 53
  gastric loss, in metabolic alkalosis of children, 392
  gastric transport mechanism, 256
  methanol mixture, fatty acid esterification with, 114, 115
  -nitric acid soln., electrode decoating of Sunderman conductivity apparatus, 172, 173
  soln. for total base detn. by conductivity method, 172
Hydrocortisone, 9-alpha-fluoro-, vacuolar nephropathy due to, 312
Hydrogen ion
  gastric transport mechanism, 256
  loss, in intestinal obstruction, 333
  transfer among body fluid compartments in acid-base disturbances, 265, 266
Hydrogen ion activity, scales, evolution, 143, 144
Hydrogen ion concentration
  of blood
    in anesthesia, 344-346
    clinical considerations, 135-137, 155
    serum pCO_2 interpolation from, 128-134
  of body fluids, in acute acid-base disturbances, 265-272
  Cl shift response to, 254, 255
  detn. by electrometric method
    standard buffers for, 151-153
    temp. dependence, 146-148, 151
  gradients, of body fluid compartments, 270
  scale evolution, 143, 144
  serum ultrafiltration response to, 66
pH, the notation, origin, 143
pH meter
  calibration, 153
  circuit diagram, 154
  electrode system standardization, 153, 154
  principle, 148, 149
  types, 149, 150
Hydrogen peroxide
  mixture with potassium ferricyanide for serum chromogen oxidation, 39
  Na detn. with, 11
  SO_4 detn. with by benzidine method, 91
Hydrogen scale, definition, 144
Hydrogen sulfide, S utilization from, 260
Hydronephrosis, hypokalemia, 312, 317
p-Hydroxdiphenyl, *See* Phenol, p-phenyl-.
N-Hydroxynaphthalimide method for Ca detn.
  apparatus, 53
  calculations, 55
  calibration curve, 53, 54
  general aspects, 42, 43, 45, 51
  principle, 51
  procedure, 54, 55
  reagents, 51-53
Hypercalciuria, renal parenchymal calcification in, 321
Hypermia, in Mg deficiency, 323
Hyperoxaluria, serum oxalic acid level in, 106
Hyperpyrexia, respiratory alkalosis in, 137
Hypertension
  in aldosteronism, 229, 230
  malignant
    K depletion in, 312
    with terminal uremia, serum composition in, 302
  Na and K balance in, 307
Hyperventilation syndrome renal parenchymal calcification in, 321
Hypotension, in liver disease, 355
Hypothalamic-neurohypophyseal system, water metabolism regulation by, 274, 275

Hypothermia
  hypermagnesemia in, 58
  serum electrolyte patterns in, 293-298

I

Ileum, Cl active transport, 254
Inanition, serum electrolyte patterns in, 293-296, 298
Indicators
  adsorption, Cl detn. with, 75
  for Ca detn., 52, 53
  for Cu detn., 37-42, 44
  ferric, in Cl detn., 74
Indole, S conjugation with, 261
Infants
  electrolyte disturbances, 388-393
  fluid and electrolyte balance in infectious diseases, 392, 393
  hypernatremia, clinical picture and therapy, 388-391
  metabolic alkalosis
    clinical picture and therapy, 391, 392
    due to persistent emesis, 391-392
  premature, hypernatremia, 389
  renal lesions in Na and Cl imbalance, 319
  serum pCO_2 values by electrometric method, 141
  serum P level, 103
  serum K values, 30
Infection
  Cl metabolism in, 350
  as complication of burn trauma, 399
  fluid and electrolyte balance of children in, 392, 393
  prolonged, lowered serum Mg in, 250
  renal K depletion and, 312
Inheritance
  of familial periodic paralysis, 374
  of paramyotonia congenita, 373, 374
Insulin
  hypokalemia due to, 29
  paretic episode precipitation by in familial periodic paralysis, 378
  responsiveness to, fluid and electrolyte loss impairment of, 335-337
  as S source, 260
  S incorporation in, 261
Interstitial fluid
  body per cent composition by weight, 3
  interchange with blood, 7
  organic acid changes in ketoacidosis, 105
Intestine
  anomalies, obstruction due to, 332
  Cl conservation, 253
  Cl exchange with external environment, 256
  disease, vacuolar nephropathy in, 310, 311
  fluid and electrolyte transport during obstruction, 332-336
  high obstruction
    features, 333
    renal parenchymal calcification in, 321
  low obstruction, features, 334
  mucosa, functional response to obstructive distention, 333, 335
  obstruction
    fluid and electrolyte changes in, 332-336
    lowered serum Mg in, 250
    types, 333
  postoperative drainage, lowered serum Mg due to, 250
  small, obstruction causes and frequency, 332
  Na-dependent non-electrolyte transport, 233, 235, 236, 238-240
  Na transport mechanism, damage, salt depletion due to, 280
  tube drainage and K loss, 29
  ulceration, due to K toxic effects, 310
Intoxication, as complication of burn trauma, 399
Intracellular fluid
  body per cent composition by weight, 3
  electrolyte components and vol. in diabetes, 364, 365
  electrolyte levels in, normal values, 4, 5
  pH, in acute acid-base disturbances, 265-272
  osmolality, in heart failure, 289
  K level and regulation, 20
Intussusception, intestinal obstruction due to, 332
Iodine
  protein-bound
    detn. by X-ray spectroscopy, 225
    liberation in X-ray spectroscopy, 213, 214
Iodocyanine green, plasma clearance in liver function assessment, 359
Ion exchange resins
  Ca and Mg separation by, 57, 58
  in Na detn., 9, 10
Iron
  detn. by X-ray spectroscopy, 218, 219, 225
  level in blood, values by X-ray spectroscopy, 225, 226
  level in bone, 368, 370
  standard soln. for X-ray spectroscopy, 211, 218, 220
Iron phosphate, K loss to in total base detn., 165
Irritability, in hypernatremia of children, 390
Irving-Watts method for Ca and Mg detn., 43
Itano method for Cl detn., 76
Iwaski method for Cl detn., 76

J

Jacobs-Hoffman method for K detn., 23, 24
Jackson-Breen-Chen method for Ca detn., 39

Jackson-Brown method for Ca and Mg detn., 40
James-Martin fatty acid separation by gas chromatography, 113
Jarrell-Ash atomic absorption apparatus, modifications for Sunderman-Carroll method for serum Ca and Mg, 202, 204
Johnson observation on K precipitate solubility, 20
Jones-McGuekin method for Ca detn., 39, 40

K

Kendall's amorphous adrenal fraction, 228
Kerr method for K detn., 23
Kessler-Wolfman method for Ca and P detn., 41
Ketoacidosis, organic acid metabolism in, 105
Ketone bodies
  detn., in blood and serum, 110, 111
  detn. in ketoacidosis, 105
  detn. by salicylaldehyde-acetone method
    apparatus, 110
    blood and serum adaptation, 110, 111
    general aspects, 109
    principle, 109, 110
    reactions, 110
    reagents, 110
  detn. in urine, 112
  detn. and diagnostic significance in serum, 104
  formation, anesthesia depth response to, 341
  level in blood, value range, 112
  level in urine, value range, 112
Ketosis
  diabetic
    fluid and electrolyte changes in, 362
    serum keto acid levels in, 104
  in starvation, 112
Kidneys
  acid elimination in acidosis, 136
  aldosterone action on, site, 228, 229
  aldosterone secretion regulation by, 228
  antidiuretic hormone effects on, review, 275
  biochemical and histochemical changes in K depletion, 315, 316
  Ca excretion, sex steroid effects on, 244
  Ca metabolism control, 245
  Ca and P excretion in hyperthyroidism, 243, 244
  Ca and P tubular reabsorption
    cortical steroid effects on, 244
    parathhormone effect on, 244
  capsule, partietal cells, vacuolation in hypokalemia, 311
  changes in K replenishment, 317, 318
  Cl conservation, 253
  Cl transport, 256, 257
  cortex
    necrosis, parenchymal calcification in, 321
    Na-dependent non-electrolyte transport, 233
  disease
    Ca metabolism in, 308, 309
    Cl metabolism in, 308
    electrolyte alterations in, 299-331
    electrolyte metabolism in, 306-309
    hypernatremia of children and, 389
    Mg metabolism in, 309, 322
    PO_4 metabolism in, 308
    K metabolism in, 307-308
    serum organic acid level in, 105
    Na metabolism in, 306-307
    SO_4 metabolism in, 308
  diuretic effects on in K depletion, 265
  dysfunction, in liver disease, 353
  enzyme activity, in hypokalemia, 315, 317
  failure, acidosis in, 106
  fibrosis, in hypokalemia, 312, 317
  free-water clearance, 273, 274
  function
    aldosterone vs. medullary amine effects on, 228, 229
    in antidiurectic hormone secretory abnormality, 276
    in diabetes, 363
    in heart failure, 288-292
    in hypernatremia of children, 389, 390
    in metabolic alkalosis of children, 391, 392
    pitressin effects on, 277
    in renal insufficiency, 299
    in salicylate poisoning, 402, 403
    serum K values and, 29, 35
  glomeruli
    filtration rate in K depletion, 318
    permeability increase due to burns, 398
  hexose monophosphate system in Na and Cl imbalance, 321
  hyaline droplets
    in hypernatremia and hyperchloridemia, 319
    in hypokalemia, properties, 310, 311, 313-315
    in Na and Cl imbalance, 319
  hyperplasia, in hypokalemia, 316
  hypertrophy, in K depletion, 316
  infection, K depletion and, 312, 317
  insufficiency
    acute and chronic, serum electrolyte changes in, 299-302, 305
    Mg toxicity in, 250
    terminal, serum electrolyte and nonelectrolyte patterns in, 301-305
  juxtaglomerular apparatus
    hyperplasia in aldosteronism, 230
    lesions in hypochloridemia and hyponatremia, 320, 321
    Na balance regulation, 339, 340
  lesions
    in adrenocortical hyperfunction, 319
    in Ca imbalance, 321

    in Cl imbalance, 319-321
    in electrolyte imbalance, 310-331
    in hyperchloridemia, 319-321
    in hypernatremia, 319, 321
    in hypokalemic alkalosis, 318
    in Mg imbalance, 322-325
    in PO_4 imbalance, 321, 322
    in K imbalance, 310-319
    in Na imbalance, 319-321
  Mg dynamics in, 249
  Mg transport, 322
  morphology, serum chemical changes and, 299
  parenchyma, calcification, associated disorders, 321
  P metabolism control, 245
  K distr. in K depletion, 316
  Na conservation, impairment in hypokalemia, 318, 319
  Na-dependent non-electrolyte transport, 235, 236
  SO_4 excretion, 262, 263
  S transport, 262
  tubules, Cl active transport, 254
  tubules, cytoplasmic vacuolization
    in bacillary dysentery, 312
    in cholangitis, 312
    in cholera, 312
    in diarrhea, 310-312
    in diverticulitis, 312
    due to chlorthiazide, 312
    due to dioxane poisoning, 310
    due to EDTA, 310
    due to 9-OC-fluorohydrocortisone, 312
    due to mannitol, 310
    due to Na and Cl imbalance, 319
    due to sucrose, 310
    in gastrointestinal disease, 310, 311
    in hydrocephalus, 312
    in hypokalemia, 310, 319
    in regional enteritis, 312
    in renal infection, 312
    in ulcerative colitis, 310
    in Whipple's disease concomitant with parathyroid hyperplasia, 312
  tubules
    dilation in hypernatremia, 319
    disease, hypophosphatemic vit. D refractory rickets in, 245
    ectasia, parenchymal calcification in, 321
    hyperplasia in hypokalemia, 315
    necrosis, in Na and Cl imbalance, 319
    K loss, 312
    salicylate direct effects on, 402
    Na and K dynamics in, aldosterone effect on, 228-231
  urine concentrating ability, in diabetes, 362-363
    urine concentrating impairment in hypokalemia, 318
Kingsley-Robnett method for Ca detn.
  adaptation to AutoAnalyzer, 41
  Brush modification, 39
  and Mg, 39
  modification of Baar's method for serum Ca, 37-39
Kirchoff, flame spectra analysis, 189, 199
Kleeman method for sulfate detn., 92
Kolthoff method for Mg detn., 56, 57
Kovács Tárnoky method for Ca detn., 39
Kóvacs-Tárnoky, method for Mg detn., 57
Kramer-Gittleman method for K detn., 23
Kramer-Gittleman method for sodium pyroantimonate iodometric analysis, 9, 10
Kramer-Tisdall method
  for K detn., 20, 23
  for Na detn., 9
Kreb's cycle, intermediates, gas chromatography, 113, 117
Kuttner-Cohen method for P detn., 99

L

Lactate
  methanol intoxication therapy with, 404
  serum level in lactic acidosis, 104
Lactation, Cl loss in, 253, 255
Lactic acid
  CO_2 detn. with in serum, 124-127
  detn. in blood and serum
    error sources, 108
    principle, 107
    procedure, 107, 108
    reagents, 107
  detn. and diagnostic significance in serum, 104
  detn. by enzyme method, 108
  interference in ketone body detn., 109
  level in serum, abnormal values, 104, 105
  metabolism, in anesthesia, 341
  oxidation in acidosis, 136
  reagent, for CO_2 detn., 125
  standard soln., prepn., 107
Lactic dehydrogenase
  activity in kidney in K depletion, 315
  lactate detn. with, 108
Lactose, Ca and P metabolism response to, 246
Lang method for Cl detn., 75
Lanthanum chloride
  SO_4 and PO_4 interference elimination with in Ca detn., 204
  urine dilution with for atomic absorption spectroscopy, 43
Laxatives, K depletion due to, 312
Lead
  electroplating for X-ray spectroscopy, 214

  - level in bone. 368
- Lead acetate, -platinic chloride soln., electrode replatinization with for Sunderman conductivity apparatus, 173
- *Lebisthes,* SO$_4$ absorption from sea water, 261
- α-Lecithinase, role in intestinal obstruction, 335
- Leeds and Northrup sugar-ash bridge, Sunderman modification for total base and Na, 169
- Leighton equation for ultrafiltrable Ca, 66, 70
- Lethargy, in hypernatremia of children, 390
- Letonoff-Reinhold method for sulfate, 91
- Leukocytes
  - count
    - high, in familial periodic paralysis, 373
    - in high intestinal obstruction, 333
  - Na-dependent non-electrolyte transport, 233, 335-336
- Levine-Cummings method for Mg detn., 57
- Liebig electrolyte studies on muscle and serum, 3
- Liebig method for Cl, 75
- Linelstrom-Diehl method for Ca detn., 40
- Lipemia, serum P level in, 226
- Lipids
  - extraction from serum for Ca detn., 39
  - interference in P detn., 97
  - osmotic effects in cardiac failure, 288
- Lithium lactate, lactate standard soln. prepn. from, 107
- Liver
  - biopsy, in disease and regenerative assessment, 360
  - disease
    - NH$_4$ intoxication in, 356
    - fluid and electrolyte metabolism in, 353-361
    - hypotension in, 355
  - disorders, serum alpha-ketoglutarate level in, 106
  - glycogenolysis, anesthesia depth response to, 341
  - glycogen storage and glucose output in familial periodic paralysis, 380, 382, 383
  - iodocyanine green clearance, 359, 360
  - Mg level, 322
  - mitochondria, in Mg deficiency, 324
  - regeneration, assessment and parameters in, 360
  - SO$_4$ oxidation to ionic sulfate, 261
- Lloyd method for sulfate detn., 92
- Lochead-Purcell method for K detn., 20
- Love method for Ca detn., 42
- Lundergardh, flame spectra analysis, 189
- Lungs
  - alveoli
    - CO$_2$ output and alveolar ventilation as related, 267
    - CO$_2$ tension, arterial blood CO$_2$ tension and, 341-342
  - Cl shift, mechanism, 255
  - disease
    - anesthesia in, 342
    - Cl metabolism in, 350
  - edema or injury, respiratory acidosis in, 137
  - massive edema due to burn trauma, 398
- Lysine, Na-dependent transport, 233, 239

M

- Macro-Coleman method for Ca detn., values, 41
- MacDougall method for K detn., 22
- Magnesium
  - acetylcholine release response to, 248
  - ATPase-dependent system, 240
  - balance, in anesthesia, 340
  - Berzelius' analysis for muscle, 3
  - binding by serum proteins, 58
  - Ca detn. in presence of, 38, 41, 42
  - Ca and P metabolism response to, 246
  - Ca separation from with ion exchange resin, 57, 58
  - cellular and extracellular complements, interference phenomena, 63
  - deficiency
    - abnormalities in, 249, 250
    - histamine release in, 323
    - symptoms, 323
  - detn., by atomic absorption spectrometry
    - apparatus, 202
    - general considerations, 43, 58, 199, 201
    - instrument parameters, 203, 204
    - interference phenomena, 204, 205, 207
    - operating conditions, 203
    - procedure, 202, 203
    - reagents, 201, 202
    - value range, 203, 205, 207
  - detn., by Bohoun method
    - error sources, 64, 65
    - value range, 65
  - by cathode ray polarography, 58
  - by chemical methods, 56, 65
  - by EDTA-dye methods, 57
  - by flame photometry
    - apparatus, 195
    - calculations, 196
    - error sources, 196
    - general aspects, 57
    - principle, 195
    - procedure, 196
    - standard and specimen prepn., 195
    - value range, 197
  - by 8-hydroxquinoline method, 56
  - by ion exchange resin methods, 57, 58
  - by magnesium ammonium phosphate method
    - calculations, 62
    - general aspects, 56, 60

    principle, 60
    procedure, 61, 62
    reactions, 60
    reagents, 60, 61
  by polarographic method, 43
  by special methods, 57, 58
  by titan yellow method, 56, 57
  by X-ray spectroscopy, 58, 218, 219
  direct precipitation from serum, discovery, 22, 23
  direct titration in serum, 40
  distr. in body, 248, 249
  enzyme activation by, 248
  exchangeable, in bone, 370
  forms and functions in body, 248, 249
  imbalance, renal lesions in, 322-324
  interference in Ca detn., 38
  interference in Cu detn., 37
  intoxication, manifestations, 250
  level in bone, 368, 370
  level in body fluids and tissues, 322
  level in serum
    reported values, 4, 5, 58, 150
    in starvation, 296
    in uremia, 302
    value range by atomic absorption spectrometry, 203, 205, 207
    value range by flame photometry, 197
  loss
    in burn trauma, 399
    in diabetes, 362, 363, 365
  metabolism
    derangements, 65
    in lobar pneumonia, 351
    in renal disease, 309
    regulation, 248
  neuromuscular excitability response to, 248
  nutritional requirements, 249
  removal for Na detn., 8
  renal transport, 322
  soluble complexes in plasma, 249
  spectral line in emission photometry, 195
  standard solns., prepn., 63, 64, 195, 201, 202
  total serum, ultrafiltrable Mg detn. from, 66, 69-73
  ultrafiltrable, detn. in serum
    apparatus, 67
    calculations, 69-72
    principle, 66
    procedure, 68
    normal values for serum, 69
Magnesium ammonium phosphate
  method for Mg detn.,
    calculations, 62
    general aspects, 56, 60, 62
    principle, 60
    reactions, 60
    reagents, 60, 61
Magnesium hydroxide
  interference in Ca detn., elimination, 37
  titan yellow reaction with in Mg detn., 57
Magnesium phosphate, ubiquitousness in animal bodies, 3
Magnesium pyrophosphate, Mg detn. as, 56
Magnesium sulfate
  Ca photometric detn. with, 41
  excretion, values, 263
  S utilization from, 260
  toxic manifestations in renal insufficiency, 250
Malaria, Cl excretion in, 350
Malmstadt-Hadviioannow method for Ca detn., 38
Malmstadt-Weinfordner potentiometric Cl detn. method, 76
Malnutrition
  endemic, in Egypt, blood Zn level in, 226
  lowered serum Mg in, 250
Mammary gland, Cl loss from, 253, 255
Manganese, level in bone, 368
Mannitol
  renal tubular vacuolization due to, 310
  serum osmolarity modification by, 288
  water and electrolyte response to in heart failure, 290
Mann-Yoe reagent for Mg, Bohoun application, 57, 62-65
Manometry, Ca detn. by, 43
Marbiott method for inorganic P and Mg detn., 23
Martin-Synge gas chromatography innovation, 113
Mast cells, degranulation in Mg deficiency, 323
Mathies-Lund method for Ca detn., 43
Menstruation, Cl loss during, 253
Maruna method for Na detn., 11
McAllistor-Yarbro method for Ca detn., 38
McChance-Shipp method for Na detn., 12
McCrudden method for Ca detn., 43, 44
McLean-Hastings equation for ultrafiltrable Ca, 66, 69-70
McLean's nomogram for Henderson-Hasselbalch equation soln., 132, 133
McLean-Van Slyke method for Cl detn. in serum, 75
Melanin, S incorporation in, 261
Membranes
  ATPase in, role in active transport, 240
  biological, structure and function, 233, 234, 235
  ion transport, Ca role in, 242
  semi-permeable, for serum osmolarity detn., 183
Mercuric chloranilate method for Cl detn.
  evaluation, 83-86
  general survey, 75
  innovation and modification, 76

  - principle, 77
  - reagents, 77, 78
  - routine method, 78
  - serum adaptation, 77-79
  - sweat adaptation, 79
  - ultra micro-modification, 79
  - urine adaptation, 79, 80
- Mercuric chloranilate reagent, prepn., 77, 78
- Mercuric chloride, Cl detn. as, 75
- Mercuric nitrate, Cl detn. with, 75, 80, 81
- Mercuric thiocyanate, Cl detn. with, 76
- Mercurimetric method for Cl detn.
  - calculation, 81
  - evaluation, 83-86
  - general survey, 75
  - principle, 80
  - procedure, 81
  - reagents, 80, 81
- Mercury
  - blood deproteinization with for SO_4 detn., 90
  - detn. by X-ray spectroscopy, 217, 218
  - electroplating for X-ray spectroscopy, 214
- Mercury bichloride, poisoning
  - renal parenchymal calcification in, 321
  - serum electrolyte patterns in, 300, 301
- Mercury compounds
  - diuretic action
    - Cl imbalance due to, 258
    - K depletion due to, 265
- Mercury-diphenylcarbazone coordination complex
  - formation, 80
  - properties, 75
- Metals
  - electroplating, for X-ray spectroscopy, 214
  - heavy, detn. by X-ray spectroscopy, 218, 225
  - trace, detn. by X-ray spectroscopy, 225
- Methane, diazo-, fatty acid esterification with, 114, 115
- Methanol
  - contaminants, implication for toxicity studies, 404
  - -HCl mixture, fatty acid esterification with, 114, 115
  - intoxication
    - serum electrolyte patterns in, 404, 405
    - serum formic acid level in, 105
    - therapy, 404
  - oxidation, ethanol inhibition of, 404
  - oxidation products, toxic effects, 404
- Methionine
  - Na-dependent transport, 233, 239
  - as S source, 260
- Micro-buret, for Ca detn. by Clark-Collip method, 48
- Micro-Coleman method for Ca detn., values, 41
- Migraine, in familial periodic paralysis, 374
- Milk, Cl loss in, 253, 255
- Milk-alkali syndrome, renal parenchymal calcification in, 321
- Mitochondria, respiration in acid-base disturbances, 268-270
- Molybdate method for P detn.
  - calibration, 102
  - evaluation, 102
  - error sources, 103
  - principle, 101
  - procedure
  - reagents, 101, 102
  - value range, 103
- Molybdate reagent, for P detn., prepn., 101
- Molybdenum blue
  - formation in cobalti-nitrite reaction for K., 27
  - phosphomolybdic acid reduction to in Mg detn., 56, 60, 62
- Molybdic acid
  - complexes, reduced, absorption spectra, 98
  - reduction, P catalysis of, 97, 98
- Montgomery method for Mg detn., 57
- Mori method for Ca detn., 40
- Moseley, X-ray emission studies, 209
- Mouth, dryness, osmotic activity control by, 274
- Mucin, S utilization from, 230
- Muraca-Bonsack method for Na detn., 12
- Murexide
  - Ca detn. with, 37, 40, 43, 44
  - Mg detn. with, 57
  - stability in propylene glycol, 37
- Muscle
  - cation values in familial periodic paralysis, 376
  - characteristics, in hypernatremia of children, 389
  - electrolyte studies on, historical aspects, 3
  - endoplasmic reticulum, water loculation in familial periodic paralysis, 376, 377, 378, 384
  - glycogen storage and glucose output in familial periodic paralysis, 379, 380, 382, 383
  - pH, in acid-base disturbances, 269
  - metabolic rate, in familial periodic paralysis, 383, 384
  - mitochondria
    - in familial periodic paralysis, 377, 379, 383, 384
    - in sporadic periodic paralysis of thyrotoxicosis, 383
  - PAS-positive substance in familial periodic paralysis, 376, 383
  - K influx in familial periodic paralysis, 375, 376
  - K level
    - normal values, 20
    - in paramyotonia congenita, 387
    - in K depletion, 316
  - respiratory, paralysis and hypokalemia, 29
  - resting potentials in familial periodic paralysis, 375, 376, 383, 385

  - stimulation refractoriness in familial periodic paralysis, 374, 376
  - striated
    - lesions, in Mg deficiency, 323
    - Mg level, 322
  - ultrastructure, in familial periodic paralysis, 377
  - vacuolization in paramyotonia congenita, 386
  - water and electrolyte composition in familial periodic paralysis, 376, 381-383
- Myeloma
  - multiple
    - renal parenchymal calcification in, 321
    - serum composition in, 302
- Myxedema
  - bone electrolyte metabolism in, 372
  - Ca and P metabolism in, 244

N

- Nalefski method for sulfate, 92
- 2, 7-Naphthalenedisulfonic acid, 3-[(5-chloro-2-hydroxyphenyl) azo]-4, 5-dihydroxy-, Na salt, *See* C.I. Mordant Blue 13
- Naphthalhydroxamic acid, *See* N-Hydroxynapthalmide
- 2-Naphthol-4-sulfonic acid, 1-amino-
  - phosphomolybdate reducing reagent prepn. with, 101, 102
  - P detn. with, 98
- Naphthoquinone-4-sulfonate, SO_4 detn. with, 91, 94
- 1-Naphthylamine, K detn. with, 23
- Narcosis, electrolyte metabolism in, 337-348
- Natelson microgasometer, models and commercial availability, 125
- Natelson microgasometric method for CO_2 detn.
  - apparatus, 125
  - calculations, 126
  - error sources, 127
  - general aspects, 124
  - principle, 124
  - procedure, 125, 126
  - reagents, 124, 125
- National Bureau of Standards pH scale, 143, 151
- National Heart Institute, fatty acid methyl ester reference standards, 114
- Negrin method for P detn., 99
- Neill-Neely method for detn., 57
- Nephelometry
  - Ca detn. by, 38
  - SO_4 detn. by, 92
- Nephritis
  - chronic, serum total base values in, 161
  - glomerular, with terminal uremia, serum composition in, 302
  - lowered serum Mg in, 250
  - K losing, 307, 318
  - Na losing, 306
  - terminal, serum composition in, 302
- Nephropathy, kaliopenic, in aldosteronism, 229
- Nephrocalcinosis, renal function impairment in, 321
- Nephrosclerosis, in hypokalemia, 312
- Nephrosis
  - hemoglobinuric, as complication of burn trauma, 399
  - lipoid
    - serum electrolyte patterns in, 300, 301
    - serum P level in, 226
  - serum total base values in, 161, 162
- Nephrotic syndrome, aldosteronism in, 230
- Nephrotoxins, renal parenchymal calcification due to, 321
- Nerve cells, dehydration by anesthetics and narcotics, 337
- Nervous system, central
  - depression, in methanol intoxication, 404
  - disease
    - electrolyte abnormalities in, 277, 278
    - hypernatremia of children and, 389, 390
  - lesions
    - in Mg deficiency, 323
    - in NaCl intoxication, 319
  - Cl transport regulation, 256
  - milk composition regulation, 255
  - sympathetic stimulation, anesthesia depth response to, 341
  - K level, normal values, 20
- Neuromuscular system
  - abnormalities, in Mg deficiency, 249, 250
  - excitability, Mg role in, 248
  - irritability, Ca role in, 242, 248
  - lesions, in Mg deficiency, 323
  - Mg excess effects on, 250
- Neutron activation analysis
  - in K detn., 25
  - in Na detn., 9
- Newborn, serum K values, 30
- Nickel, detn. by X-ray spectroscopy, 218
- Nitrate, salivary gland electropotential response to, 256
- Nitric acid
  - deproteinizing reagent prepn. with, 77
  - -HCl soln., electrode decoating of Sunderman conductivity apparatus, 172, 173
  - soln., for Cl detn., 81
- Nitrogen
  - balance
    - in burn trauma, 399, 400
    - in diabetes, 364
    - in febrile states, 297
    - in infectious diseases of children, 292

  - as carrier gas for fatty acid chromatography, 114
  - non-protein
    - level in serum in renal insufficiency, 300-303
    - level in serum in uremia, 302
    - standard soln. for NH_3 detn., 120
    - urea, plasma osmolality calculation from, 341
- Non-electrolytes
  - active transport
    - ATPase role in, 240
    - ouabain inhibition of, 239
    - Na effect on, 238
  - carrier-mediated diffusion, Na effect on, 237, 238
  - directional fluxes, Na control, 236
  - distr., regulation, 6, 7
  - osmolality, 6
  - patterns in serum in uremia, 301-305
  - transport
    - impairment, clinical significance, 240, 241
    - Na role in, 233-241
    - Na transport as related, 239, 240
- Norepinephrine, renal function response to, 229, 231
- Nuclear Fast Red, Ca detn. with, 37, 38, 44
- Nucleic acid, deoxyribo-, hepatic formation in regenerative assessment, 360
- 5-Nucleotidases, activity in kidney depletion, 315
- Numbness, in metabolic alkalosis of children, 391

O

- Obesity, body constituent variations in, 338
- Octyl alcohol, CO_2 detn. with, 125-127
- Oesper-Pauerstine method for Na detn., 10
- Oliguria
  - in burn trauma, 396
  - hyperkalemia in, 29, 35
  - in hypernatremia of children, 389, 390
- Onsager equation for serum conductivity value corrections, 169, 170
- Opium, derivatives, osmoregulatory system response to, 275
- Osmometer, thermoelectric
  - development, 183
  - principle, 181-183
  - serum osmolality detn. by
    - error sources, 187
    - instrumentation, 184-187
    - literature review, 183, 184
    - principle, 181-183
    - procedure, 187
    - standard solns., 186
    - value range, 187
- Osmotic pressure
  - of biological fluids, detn.
    - by freezing point method, 176-181
    - principles, 6
    - survey of methods, 183
  - of body fluids
    - equilibrium maintenance, 5, 6, 288, 289
    - in heart failure, 288-292
    - regulation, 273-275
  - of burned tissue, 396
  - neural regulation, 274, 275
  - regulation, 273-275
  - of serum, indices, 288
- Osteitis
  - deformans, renal parenchymal calcification in, 321
  - fibrosa
    - of primary nutritional origin, 245
    - of renal origin, 245
- Osteomalacia, in sprue, 245
- Osteoporosis
  - electrolyte metabolism in, 371
  - in hyperadrenocorticism, 244
  - of primary nutritional origin, 245
  - renal parenchymal calcification in, 321
- Ouabain, active transport inhibition by, 239
- Ovomucoid, S utilization from, 260
- Owen-Verner Syndrome, renal parenchymal calcification in, 321
- Oxalate
  - Ca precipitation for flame photometric detn., 42
  - interference in P detn., 103
- Oxalate-permanganate method for Ca detn.
  - apparatus, 49
  - calculations, 50
  - normal values, 51
  - principle, 48
  - procedure, 49, 50
  - reagents, 48, 49
- Oxalic acid
  - $KMnO_4$ bleaching with in cobalti-nitrite reaction for K, 21, 22
  - level in serum
    - diagnostic significance, 104
    - in hyperoxaluria, 106
  - level in serum and urine, interrelationship, 106
  - poisoning, hypermagnesemia in, 250
- Oxalosis
  - renal parenchymal calcification in, 321
  - urine precipitation with for Ca detn. by AutoAnalyzer, 40
- 2,4-Oxazolidinedione, 5,5-dimethyl-, intracellular pH detn. with, 269, 270
- Oxidimetric method for Na detn., 10, 11
- Oxygen
  - pulmonary insufflation, metabolic effects, 345
  - tension, in anesthesia, 341-347

P

- Paget's disease, of bone, electrolyte metabolism in, 371
- Pain, osmoregulatory system response to, 275
- Pancreatic juice, Cl transport to gastric juice, 256
- Pancreatitis, lowered serum Mg in, 250
- Papaverine, respiratory response to in anesthesia, 342
- Paper pulp, potassium sodium cobalti-nitrite precipitate filtration on, 23
- Pappenhagen-Jackson method for Ca detn., 38
- Paracentesis, plasma vol. response to, 357, 358
- Paraldehyde, seizure control with in hypernatremia of children, 391
- Paralysis
  - in aldosteronism, 229
  - familial periodic
    - carbohydrate metabolism abnormality in, 373, 378-385
    - cation values and kinetics in, 376
    - electrolytes in, 373-385
    - episodes, KCl in prophylaxis of, 375
    - inheritance, pedigree, 374
    - metabolic rate in, 383, 384
    - muscle PAS-positive substance in, 376, 383
    - muscle resting potentials in, 375, 376
    - muscle stimulation refractoriness in, 374, 376
    - muscle water uptake in, 376, 377
    - nocturnal episodic nature, 373, 377
    - P metabolism in, 373, 382
    - K metabolism in, 373, 375-377, 381-384
    - prophylaxis with exercise, 373, 379, 383
  - hypokalemia and, 29
  - respiratory, alkalosis in, 137
  - sporadic periodic, in thyrotoxicosis, 383, 384
- Paramyotonia congenita
  - carbohydrate metabolism in, 385-387
  - characteristics, 385
  - cold precipitating effects in, 386, 387
  - deep tendon reflex depression in, 387
  - electrolytes in, 385-387
  - eyelid lag in, 386
  - facial muscle paresis in, 385, 386
  - inheritance, 373, 374, 385, 386
  - muscle fiber vacuolization in, 386
  - K metabolism in, 373, 386, 387
  - serum Ca level in, 386
  - tongue myotonia in, 385, 386
- Parathormone
  - bone and renal lesions due to, PO_4 loading as intensifying, 322
  - Ca and P metabolism response to, 244, 245
- Parathyroid gland
  - Ca and P metabolism control by, 244, 245
  - disease, electrolyte metabolism in, 368-372
  - hyperfunction
    - lowered serum Mg in, 250
    - in Mg deficiency, 325
    - renal parenchymal calcification in, 321
  - hyperplasia, concommitant with Whipple's disease, vacuolar nephropathy in, 312
- Parenteral fluids
  - burn trauma therapy with, 399
  - for fluid and electrolyte balance maintenance in infections, 392
  - in hypernatremia of children, 389-391
  - for metabolic alkalosis therapy in children, 392
  - prolonged admn., serum lowered Mg due to, 250
- Paresthesia, in aldosteronism, 229
- Parker serum standards for Ca photometric detn., 41
- Parr apparatus for deproteinization, 74
- Patton-Reeder method for Ca detn., 38
- Pediatrics, electrolyte metabolism disturbances in, 388-393
- Perchloric acid
  - Ca detn. with, 42, 43
  - P detn. with, 98
  - serum filtration with for Mg photometric detn., 57
- Pericarditis, serum total base values in, 616, 162
- Perivasculitis, in Mg deficiency, 323
- Permanganate, SO_4 detn. with, 91
- Peters-Van Slyke review of K methods, 24
- Peters-Van Slyke values for total serum base, 158
- Pharynx, dryness, osmotic activity control by, 274
- Phenformin, lactic acidosis and, 105
- Phenol
  - p-aminomethyl-, as reducing agent for P detn., 98, 99
  - burn therapy with, 394
  - derivatives, S conjugation with, 261
  - p-phenyl-
    - acetaldehyde condensation product with in lactate detn., 107, 108
    - reagent for lactate detn., prepn., 107
  - soln., for NH_3 detn., 120
- Phenolphthalein, indicator for total base detn., 163, 164
- Phenol reagent, for K detn. by cobalti-nitrite method, 28
- p-Phenylenediamine, N-phenyl-, HCl, phosphomolybdate reduction with, 99
- Phosphatase
  - activity in blood and tissues in Mg deficiency, 324
  - activity in kidney in K depletion, 314, 315
  - Mg activation, 248
- Phosphate
  - detn.
    - by AutoAnalyzer method, 41
    - by automatic methods, 99
    - general aspects, 97

    - by Greenwald composite method, 22
    - interference phenomena, 97, 102, 103
  - detn. by molybdate method
    - calibration, 102
    - error sources, 103
    - evaluation, 102
    - normal values, 103
    - principle, 101
    - procedure, 102
    - reagents, 101, 102
  - energy-rich, requirements for intestinal water transport, 334
  - excretion, in lobar pneumonia, 350
  - exchangeable, in bone, 370
  - imbalance, renal lesions in, 321-322
  - inorganic, K titration response to, 23
  - interference in Ca detn., 38, 41, 42, 204, 205, 207
  - in Na detn., 8, 9
  - in SO_4 detn., uranium acetate elimination of, 91, 92
  - in total base detn., elimination, 163, 164
  - level in blood, decrease in familial periodic paralysis, 373
  - in serum
    - hyperventilation as decreasing, 341
    - in renal insufficiency, 300-301
    - reported values, 4, 5, 103, 159
  - loss
    - in diabetes, 363, 364, 366
    - during tissue catabolism, 297
  - metabolism
    - in anesthesia, 341
    - Mg intake effect on, 322
    - in K depletion, 318
    - in renal disease, 308
  - molybdic acid reduction catalysis by, 97, 98
  - standard solns., prepn., 61, 102
  - SO_4 detn. response to, 91
  - ubiquitousness in animal bodies, 3
- Phosphate buffer
  - standard
    - composition, 152
    - pH values and temp. correction, 151
- Phosphine generator, for sample prepn. in X-ray spectroscopy, 213, 214
- 6-Phosphogluconate dehydrogenase, activity in kidney in Na and Cl imbalance, 321
- Phospholipids, level in blood, P level as related, 226
- Phosphomolybdate
  - reducing agents for, 97-99
  - reduction
    - with hydrazine sulfate, 99
    - with hydriodic acid, 99
    - with stannous chloride, 99
- Phosphomolybdate method for P detn., 97
- Phosphomolybdic acid
  - reduced, absorption spectrum, 98
  - reduction to molybdenum blud in Mg detn., 56, 60, 62
- Phosphomolybdic acid complex
  - aminonaphtholsulfonic acid reduction of, 98
  - ascorbic acid reduction of, 99
  - methylaminophenol reduction of, 98
  - reduction, initial description, 97
- Phosphoric acid, concentrated, deproteinizing reagent prepn. with, 77
- Phosphorus
  - detn. by X-ray spectroscopy, 214, 223
  - forms and functions in biological systems, 242, 243
  - inorganic, direct precipitation from serum, discovery, 22, 23
  - interference in Ca detn. by flame photometry, 194, 195
  - level in blood, values by X-ray spectrometry, 225, 226
  - level in bone, 368
  - level in serum
    - in familial periodic paralysis, 373, 382
    - in uremia, 302
  - loss, in burn trauma, 399
  - metabolism
    - control, general aspects, 242, 243
    - endocrine regulation, 243-245
    - gastrointestinal regulation, 245
    - nutritional factors in, 246
    - renal regulation, 245
  - role in glucose metabolism, 366
  - standard soln. for X-ray spectroscopy, 211, 218
- Phosphorylase, role in glycogenolysis in familial periodic paralysis, 379, 380
- Phosphorylation, oxidative, uncoupling in Mg deficiency, 324
- Phosphovandomolybdate complex, description, 99
- Photometer
  - Coleman model 21, correction for interference phenomena in Ca detn., 41
  - flame, with automatic background-subtraction feature, 41
- Photometry
  - Ca detn. by, 37, 38
  - emission, theory, 189, 190
  - flame
    - cation detn. by, 189-198
    - development, 189
  - flame, Ca detn. by
    - apparatus, 194
    - error sources, 194, 195
    - general aspects, 41-43, 45
    - principle, 193
    - procedure, 194

    reagents, 194
    standard soln., 194
    value range for serum, 195
  flame, Mg detn. by
    apparatus, 195
    calculations, 196
    error sources, 196
    general aspects, 57
    principle, 195
    procedure, 196
    standard and specimen prepn., 195
    value range for serum, 197
  flame, K detn. by
    apparatus, 192, 193
    error sources, 193
    principle, 192
    procedure, 193
    range of values, 30, 197
    standard solns., 192
  flame, Na detn. by
    apparatus, 191
    error sources, 191, 192
    general aspects, 9
    principle, 190
    procedure, 191
    standard solns., 190, 191
    value range for serum, 196
Phthalate buffer
  standard
    composition, 152
    pH values and temp. corrections, 151
Pictric acid, burn therapy with, 394
Pictol, *See* Phenol, p-aminomethyl-
Pitressin, water and electrolyte abnormality assessment with, 276, 277
Pituitary body
  Ca and P metabolism control by, 243
  function, serum sulfation factor and, 262
  posterior
    ethanol effects on, 275
    water metabolism regulation by, 274, 275
Plasma membrane, structure and function, 234
Plasmo Corinth B, *See* C.I. Mordant Blue 13
Platinic chloride, -lead acetate, electrode replatinization of Sunderman conductivity apparatus, 173
Platinum, platinated wire or foil, as H_2 gas electrode, 143
Pneumonia
  lobar
    Cl retention in, mechanism, 349-352
    serum electrolyte patterns in, 293, 296-298, 349-352
    with terminal uremia, serum composition in, 302
Pneumonitis, serum total base values in, 161, 162
Polarography
  Ca detn. by, 38
  cathode ray, Mg detn. by, 58
  serum Ca and Mg detn. by, 43
Polydipsia, in aldosteronism, 229
Polyuria
  in aldosteronism, 229
  Cl and K loss in, 257
Polyvinyl alcohol, Mg detn. with in titan yellow method, 57
Porphyria
  acute, lowered serum Mg in, 250
  acute intermittent, water metabolism in, 277, 278
Potassium
  balance
    in burn trauma, 399
    in hypernatremia of children, 389
    in hypertension, 307
    in metabolic alkalosis of children, 391, 392
  deficiency
    in aldosteronism, 229, 230
    carbohydrate metabolism response to, 366
    Mg deficiency additive effects with, 323
  depletion
    in adrenocortical hyperplasia, 312
    aggrevation of renal lesions due to PO_4 loading, 322
    in aldosteronism, 312
    alkalosis in, 318
    HCO_3-1 renal transport impairment in, 318
    body fluid pH in, 265, 270
    causes, 312
    in cystinosis, 312
    in diarrhea, 312
    due to enemas, 312
    due to laxatives, 312
    in emesis, 312
    in Fanconi syndrome, 312
    glomerular filtration rate in, 318
    in hydronephrosis, 317
    in malignant hypertension, 312
    organic acid excretion in, 318
    in pyelonephritis, 317
    renal biochemical and histochemical changes in, 315, 316
    renal fibrosis in, 317
    renal hypertrophy in, 316
    in renal infection, 312, 317
    renal lesions in, 310-319
    renal PAH transport in, 318
    renal tublar hyperplasia in, 315
    in renal tubular acidosis, 312
    in salicylate poisoning, 402, 403
    Na conservation impairment in, 318, 319
    in steatorrhea, 312
    in thyrotoxicosis, 312
    in ulcerative colitis, 311, 312
    uremia in, 318
    urine concentrating impairment in, 318

  - in Zollinger-Ellison disease, 312
- detn., chemical methods, 19-35
- detn. by chloroplatimate method
  - apparatus and reagents, 32
  - calculations, 33
  - general aspects, 25
  - principle, 31
  - procedure, 32
  - range of values, 30
- detn. by cobalti-nitrite method, 22, 27-31
  - range of values, 30
- detn. by flame photometry
  - apparatus, 192, 193
  - error sources, 193
  - principle, 192
  - procedure, 193
  - range of values, 30, 193
  - Na interference in, 193
  - standard solns., 192
- detn. by Greenwald composite method, 22
- detn., initial attempts, 22
- detn. by neutron activation analysis, 25
- detn. by tetraphenylboron method
  - calculations, 35
  - general aspects, 25
  - micro-modification, 35
  - principle, 33
  - range of values, 30
  - reagents, 33, 34
- detn. by X-ray spectroscopy, 223
- diffusion from cells to serum, as error source in total base detn., 165
- distr. in body fluids, 29
- distr. in kidney, in K depletion, 316
- distr. and properties, 19, 20
- excretion, in familial periodic paralysis, 376, 381
- imbalance in liver disease, 356
- imbalance, renal lesions in, 310-319
- incomplete precipitation in diluted serum, 21, 25
- intake, in familial periodic paralysis, 376
- interference in Ca detn. by flame photometry, 41, 42
- intoxication, 29, 35
- level in blood and tissues, normal values, 20
- level in bone, 363
- level in cell, relation to active transport, 238, 239
- level in erythrocytes, 35
- level in feces, in Asiatic cholera, 280
- level in feces vs. plasma in cholera, 282
- level in muscle
  - in paramyotonia congenita, 387
  - in K depletion, 316
- level in serum
  - hyperventilation as decreasing, 341
  - in infectious diseases of children, 392
  - in paramyotonia congenita, 373, 386
  - reported values, 4, 5, 29, 30, 159
  - in uremia, 302
  - value range by flame photometry, 197
  - by X-ray spectroscopy, 226
- Liebig's analysis for muscle and serum, 3
- loss
  - in cardiac failure, 289
  - in diabetes mellitus, 362, 363
  - in intestinal obstruction, 333
  - to iron phosphate, in total base detn., 165
  - during tissue catabolism, 297
  - and replenishment, 293
- metabolism
  - adrenal regulation, 228-232
  - in anesthesia, 339, 340
  - in familial periodic paralysis, 373, 375-377, 381-384
  - in lobar pneumonia, 351
  - in Mg deficiency, 324
  - in renal disease, 307-308
- muscle influx in familial periodic paralysis, 375, 376
- removal in Na detn., 8
- replenishment, renal changes in, 317, 318
- retention, in selective aldosteronism, 230
- Na displacement of in burned tissue, 398
- spectral line in emission photometry, 192
- standard soln., prepn., 27, 34, 192, 211, 218
- toxic effects on tissue, 310
- transport, in intestinal obstruction, 333, 334

Potassium chloride
- Berzelius' analysis for muscle, 3
- in electrode cell assembly for pH detn., 144, 146, 148, 153
- episodic paresis prophylaxis with in familial periodic paralysis, 375

Potassium cobalti-nitrite precipitate, solubility, 20, 21

Potassium dichromate reagent, for β-hydroxybutyrate oxidation to acetoacetate, 110, 111

Potassium dihydrogen phosphate, soln, for Mg detn., prepn., 60, 61

Potassium ferricyanide, mixture with H_2O_2 for serum chromogen oxidation, 39

Potassium hydroxide, soln., for ketone body detn., 110

Potassium iodate
- acid soln. for chloroplatinate method for K, 32, 33
- acidified, Cl detn. with, 75
- Na detn. with, 9, 10

Potassium iodoplatinate, titration with sodium thiosulfate, 33

Potassium nitrite, titration, in Clausen method for K detn., 22

Potassium periodate, in Na detn., 11

Potassium permaganate
  decomposition in Clausen modification of cobalti-nitrite reaction for K, 22
  nitrite oxidation with in alkaline, 22
  potassium sodium cobalti-nitrite reaction with, 21
  K titration with, 21, 23
  solns. for Ca detn. by Clark-Collip method, 49
  -sulfuric acid mixture, Cl detn. with, 75
Potassium phosphate, ubiquitousness in animal bodies, 3
Potassium salts, solns., potassium cobalti-nitrite precipitate solubility in, 20
Potassium silver cobalti-nitrite, precipitate analysis for K, 23
Potassium sodium cobalti-nitrate precipitate
  composition constancy, 24, 25
  control analysis, 24, 25
  wash soln. for prepn., 27
Potassium sulfate
  excretion, values, 263
  in Na detn., 9
  as standard for benzidine method for sulfate, 91
  S utilization from, 260
Pregnancy, Cl balance in, 255
Prescott observations on K precipitate solubility, 20
Pribrom method for Ca, 22
Propionic acid, level in serum, diagnostic significance, 104
Propylene glycol, murexide stability in, 37
Prostate gland, carcinoma, with terminal uremia, 302
Proteins
  balance, in burn trauma, 399
  base-bound, level in serum in uremia, 301-303
  Ca photometric detn. in presence of, 42
  Ca and P metabolism response to, 246
  catabolism, in burn trauma, 399
  deficiency, liver regeneration in, 360
  elimination for Cl detn., 74, 77-79, 83
  for SO_4 detn., 91, 92
  interference in P detn., 97, 102
  loss in renal insufficiency, 300
  metabolism, in Mg deficiency, 324, 325
  restriction, in liver disease, 359
  S-containing, metabolism, 260-261
Proteinuria, in hypokalemia, 312, 315
Prothrombin, deficiency, in salicylate poisoning, 403
Pylonephrosis
  in hypokalemia, 312, 317
  renal parenchymal calcification in, 321
Pylorus, obstruction, serum total base values in, 161, 162
Pyruvic acid
  interference in ketone body detn., 109
  level in serum, diagnostic significance, 104
  metabolism, lactate metabolism and, 105
  phenyl-, level in serum, diagnostic significance, 104
Pyruvic kinase, activity in kidney in K depletion, 315

Q

8-Quinolinol, Mg detn. with, 6

R

Radin-Gramza method for Ca. detn., 39
Reed-Davis studies on blood sulfate, 90, 91
Reducing agents, for P detn., 97, 99
Reflexes
  autonomic, osmoregulatory system response to, 275
  deep tendon, depression in paramyotonia congenita, 387
Renin, aldosterone secretion regulation by, 228, 230
Renin-angiotensin system, Na balance and, 339, 340
Respiration
  cellular
    in acid-base disturbances, 268, 269
    ADP control of, 240
  compensatory response in acid-base disturbances, 136, 137
  controlled, in anesthesia, 344
  drug effects on in anesthesia, 341, 342
  gas exchange in anesthesia, 341-345
  impaired, acid-base disturbances in, 137, 138
Respiratory center, increased sensitivity to CO_2 in salicylate poisoning, 402
Respiratory disease, blood Zn level in, 226
Resuscitation, artificial, ventrilatory efficiency, 343
Rheumatic fever, serum total base values in, 161
Rickets, hypophosphatemic-vit. D refractory, renal origin, 245
Ring oven, for sample prepn. in X-ray spectroscopy, 212
Ringer lactate, infusion, in burn trauma therapy, 397
Ritter-Spencer-Samuchson evaluation of Sulkowitch test, 43
Rosenheim benzidine method for urinary sulfate, 90
Rourke method for Na detn., 9, 10
Rubidium
  K displacement by, acid-base balance response to, 265
  renal lesion response to in hypokalemia, 317

S

Sackner-Sunderman values for total serum base, 157-161
Saifer-Kornblum method for Cl detn., 75
Salicylaldehyde, condensation product with acetone, 110
Salicylaldehyde-acetone method for ketone bodies,
  apparatus, 110
  blood and serum adaptation, 110, 111
  calculations, 111
  general aspects, 109
  principle, 109, 110
  reactions, 110
  reagents, 110
  urine adaptation, 112
Salicylate, intoxication
  analytical procedures in, 105
  early stage, respiratory alkalosis in, 137
  serum electrolytes in, 402, 403
  serum salicylate level in, 104, 105
  therapy, 403
Salicylic acid, acetyl-, intoxication, serum and serum salicylate levels in, 105
Saline
  burn therapy with, early attempts, 394
  infusion, in hypernatremia of children, 391
  isotonic, infusion, in metabolic alkalosis of children, 392
Saliva, Cl transport to gastric juice, 256
Salivary gland, Cl transport, 254, 256
Salts
  balance, sweat composition as related, 255
  depletion
    due to intestinal Na transport mechanism, 280
    due to Na transport damage, 280
Salt solutions
  hypertonic, water intoxication prevention with in acute intermittent porphyria, 278
  infusion, in burn trauma therapy, 397
  standard, for serum Na detn., 14
Sarcoidosis
  Ca enhanced absorption in, 245
  renal parenchymal calcification in, 321
Schacter method for Mg detn., 56
Schales-Schales method for Cl detn., 75, 76
Schwarzenbach, cresolpthalein complexon introduction, 41
Schwarzenbach method for Mg detn., 57
Scopolamine, respiratory response to in anesthesia, 342
Searcy-Bergquist-Tomeoni slide rule soln. of Henderson-Hasselbalch equation, 133
Seizures, in hypernatremia of children, barbiturates and paraldehyde in control of, 391
Semidine hydrochloride, *See* p-Phenylene-diamine, N-phenyl-, HCl.
Sensations, tingling, in metabolic alkalosis of children, 391
Septicemia, as complication of burn trauma, 399
Severinghaus pCO_2 electrode, principle and appln., 139-142
Sex differences
  in blood pH, 155
  in body habitus, implications for anesthesia, 337, 338
  in serum pCO_2 values, 141
Shedd method for K detn., 21
Sheehan's syndrome, serum from, SO_4 metabolism response to, 262
Sherrick method for Ca detn., 42
Shigella flexneri, diarrhea due to, mechanism, 280
Shock
  hepatic and renal lesions in, 353
  prevention in burn trauma, 395, 397
  serum lactic acid level elevation in, 105
Siggaard-Andersen method for blood collection in pH detn., 150
Siggaard-Andersen nomogram for Henderson-Hasselbalch equation soln., 133, 134
Silicic acid, columns, fatty acid preparatory treatment on for gas chromatography, 115
Silicon, molybdic acid complex, absorption spectrum, 98
Silver chloride, Cl detn. as, 74, 75
Silver chromate, Cl detn. with, 76
Silver dithizonate, Cl detn. with, 76
Silver iodate, Cl detn. with, 75
Silver nitrate, Cl detn. with, 74, 75, 82
Singer-Hastings nomogram for acid-base factors, 133
Skin
  Cl active transport, 254
  Cl loss from, 253, 255
  consistency, in hypernatremia of children, 390
  epinephrine stimulated, active Cl transport, 254
Skin glands, Cl conservation, 253
Sodium
  active transport
    Cl transport as related, 254, 255, 257
    inhibitor, in diarrhea, 280, 281
  balance
    in burn trauma, 398, 399
    in metabolic alkalosis of children, 391, 392
    regulation, 340
    renin-angiotensin system and, 399, 340
  Ca flame photometric detn. in presence of, 42
  carrier-mediated diffusion response to, 237, 238
  cation active transport response to, 239, 240
  deficiency, clinical significance, 240, 241
  detn., by chemical methods, 8-18

detn., by colorimeter method, 11, 15-18
detn. by flame photometry
  apparatus, 191
  error sources, 191, 192
  principle, 190
  procedure, 191
  standard solns., 190, 191
  value range, 196
detn. by gravimetric methods, 13-15
detn. by Greenwald composite method, 22
detn. by photometric methods, 11, 12, 18
detn. by Sunderman method, 9, 13-15
detn. by titrimetric method, 9-11
diffusion from serum to cells, as error source in total base detn., 165
energy linked transport response to, 238
excess, renal lesions due to, 319-321
exchangeable, in bone, 370
excretion, in familial periodic paralysis, 376, 381
high level in serum, pediatric aspects, 388
imbalance, renal lesions in, 319-321
intake, in familial periodic paralysis, 376
interference in Ca detn. by atomic absorption spectrometry, elimination, 204, 205, 207
interference in K detn. by flame photometry, 41, 193, 194
level in blood
  factor's conditionings, 338, 339
  normal values by conductivity method, 174
level in bone, 358
level in edema fluid in burn trauma, 397
level in feces
  in Asiatic cholera, 280
  in feces vs. plasma in cholera, 282
level in kidney, in hypokalemia, 316
level in serum
  in burn trauma, 398
  detn. from total base value, 166
  in familial periodic paralysis, 376
  in infectious diseases of children, 392
  as osmolality index in heart failure, 288, 289
  reported values, 4, 5, 159
  in starvation, 295
  in uremia, 302
  value range by flame photometry, 196
  by Sunderman conductimetric method, clinical aspects, 161
Liebig's analysis for muscle and serum, 3
loss
  in cardiac failure, 288-292
  in diabetes mellitus, 362, 363
  in lipoid nephrosis, 301, 302
  in selective aldosteronism, 230
loss and replenishment, 293
metabolism
  abnormalities, in tuberculosis, 278
  adrenal regulation, 228-232
  in heart failure, 288-292
  in liver disease, 354, 355
  in lobar pneumonia, 351
  in Mg deficiency, 324
  Mg intake effect on, 322
  pitressin effects on, 277
  in renal disease, 306-307
non-electrolyte transport role, 233-241, 236, 237
plasma osmolality detn. from, 341
-K ratio, in cobalti-nitrite precipitate, 24-25
K replacement by in burned tissue, 398
renal conservation in K depletion, 318, 319
retention
  in aldosteronism, 229, 230
  in burn trauma, 396
  postoperative, 339
spectral line in emission photometry, 190
standard solns. for flame photometry, 190, 191
transport
  in intestinal obstruction, 333, 334
  mechanism, damage in diarrhea, 280, 281
  non-electrolyte transport as related, 239, 240
Sodium acetate, soln. for K detn. by cobalti-nitrite method, prepn., 27
Sodium bicarbonate
  body fluid compartment pH changes due to infusion of, 265-270
  methanol intoxication therapy with, 404
  -NaCl soln., pCO_2 electrometric detn. with, 139, 140
Sodium bisulfite, soln., for P detn., 101
Sodium borate
  -sodium hydroxide soln., for sulfate detn., prepn., 94
  soln. for Mg detn., 63
Sodium carbonate
  anhydrous, CO_2 standard soln. prepn. from, 124, 125
  soln., for K detn. by cobalti-nitrite method, 28
Sodium chloride
  Berzelius' analysis for muscle, 3
  -HCl soln., for Ca detn. by atomic absorption spectrometry, 201, 207
  Ca photometric detn. with, 41
N-hydroxynaphthalimide Ca reagent prepn. with, 52
  intoxication
    CNS lesions in, 319
    cystitis in, 319
    gastritis in, 319
    lesions in, 319-321
    renal lesions in, 319
  retention, in lobar pneumonia, 351
  serum dilution in for Ca photometric detn., 41
  -sodium bicarbonate soln., pCO_2 electrometric detn. with, 139, 140
  soln.

    - hypertonic, water metabolism response to, 275
    - for total base detn. by conductivity method, 172
  - standard soln., prepn., 16, 78, 80, 82
  - SO_4 detn. response to, 91
- Sodium cobalti-nitrite reactions for K detn., 27
- Sodium cobalti-nitrite reagent
  - photosensitivity, 21
  - prepn., 27
  - reactions for K detn., facilitation, 21, 22
- Sodium diphenylamine sulfonate, Na detn. with, 11
- Sodium hydroxide
  - Co precipitation with in cobalti-nitrite reaction for K, 21, 22
  - EDTA alkaline soln, prepn, with, 52
  - K detn. with, 23
  - -sodium borate soln., for sulfate detn., prepn., 94
  - soln.
    - for NH_3 detn., 120
    - for CO_2 detn., 125, 140
    - for total base detn., 163, 164
- Sodium hypochlorite, serum chromogen oxidation with, 39
- Sodium lactate
  - Berzelius' analysis for muscle, 3
  - parenteral soln., contraindication in lactic acidosis, 105
  - in hypernatremia of children, 391
- Sodium molybdate, P detn. with, 98
- Sodium nitrite, titration in Clausen method for K detn., 22
- Sodium nitroprusside, soln., for NH_3 detn., 120
- Sodium oxalate, soln. for Ca detn. by Clark-Collip method, 49
- Sodium phenate, NH_3 detn. with, 119
- Sodium phosphate
  - monobasic, potentiation of bone and renal lesions due to parathhormone, 322
  - ubiquitousness in animal bodies, 3
- Sodium pyroantimonate, in Na detn., 9, 10
- Sodium salicylate, Na detn. with, 12
- Sodium sulfate
  - excretion, values, 263
  - in Na detn., 9
  - soln., hypertonic, water metabolism response to, 275
  - S utilization from, 260
- Sodium sulfite, soln., for P detn., 101
- Sodium tetraphenylboron reagent, prepn., 33
- Sodium thiosulfate
  - Cl detn. with, 75
  - standard soln. for K detn. by platinic chloride method, 32, 33
- Sodium transport inhibitor, assessment, 281
- Sodium tungstate
  - deproteinizing reagent prepn. with, 77
  - soln, for Mg detn., prepn., 63
- Soil, K detn. in, 21
- Solutes
  - concentration against electrochemical gradient, 235-237
  - hypertonic solns., water metabolism response to, 275
- Solutions, colligative properties, 6
- Solvents, alkaline murexide stability in, 37
- Somatotropin
  - Ca and P metabolism response to, 243
  - sulfation factor as related, 262
  - S incorporation in, 261
  - as S source, 260
- Sørensen-Linderstrøm-Lang scale for H ion activity, 143
- Sørensen scale for H ion activity, 143
- Spare murexide stability studies, 37
- Spectrograph
  - X-ray
    - illustrations, 221-223
    - new innovations, 224, 225
    - operation, 223, 224
- Spectrography
  - Ca detn. by, 38, 42
  - flame, historical account, 189
  - in K detn., 25
- Spectrometer
  - atomic absorption
    - burner and fuel types for, 204
    - diagram, 200
    - principle, 201, 202
  - X-ray
    - sample carrier for, 215, 216
    - sample positioning in, 210-215
- Spectrometry
  - atomic absorption
    - Ca detn. by, general considerations, 58, 199-201
    - Ca detn. by, operating conditions, 203
    - development, 199
    - Mg detn. by, apparatus, 202
    - Mg detn. by, general considerations, 199-201
    - Mg detn. by, instrument parameters, 203, 204
    - Mg detn. by interference phenomena, 204, 205
    - Mg detn. by, operating conditions, 203
    - Mg detn. by, procedure, 202, 203
    - Mg detn. by, reagents, 201, 202
    - Mg detn. by, value range, 203, 205, 207
- Spectrophotometer, for Mg detn. by Bohoun method, 64
- Spectrophotometry
  - Ca detn. by, 37-40, 42
  - ketone body detn. by, 109, 112

  lactate detn. by, 107, 108
  serum Na detn. by, 11, 15-18
  SO_4 detn. by, 92
  UV, lactate detn. by, 108
Spectroscopy
  atomic absorption
    Ca detn. by, 45
    Ca and Mg detn. by, 43
  X-ray
    Ca detn. by, 38
    Mg detn. by, 58
    serum electrolyte detn. by, 209-226
  X-ray, serum electrolyte detn. by
    apparatus, 220-223
    calculations, 224
    clinical interpretation, 226
    error sources, 225
    general aspects, 209, 210
    principle, 211-218
    procedure, 223, 224
    reagents, 218-220
    value range, 225, 226
  X-ray emission, Ca detn. by, 43, 45
Spleen, infarction, in NaCl intoxication, 319
Sprue, Ca, P and vit. D malabsorption in, 245
Sputum, Cl level in lobar pneumonia, 350
Stadie-Ross method for serum total base, adaptation from Fiske method for urinary cations, 157
Stadie-Sunderman method for serum oxmolality detn.
  apparatus, 178, 179
  error sources, 180
  principle, 177, 178
  stem corrections, 180
Stannous chloride, phosphomolybdate reduction with, 99
Starch solution, for chloroplatinate method for K., 32
Starvation
  acute, serum electrolyte patterns in, 293-298
  body fluid vol. in, 296-298
  chronic, serum electrolyte patterns in, 293-298
  ketosis in, 112
Steatorrhea, K depletion due to, 312
Steroids
  gas chromatography, 113
  S conjugation with, 262
Stomach
  Cl exchange with external environment, 255, 256
  pylorus, stenosis, hypertrophic, fluid therapy problem in children, 391
  tube drainage and K loss, 29
Stone method for Na detn., 11
Stress
  anesthesia depth response to, 341
  lowered resistance to in burn trauma, 399
Strontium
  detn. by X-ray spectroscopy, 225
  exchangeable, in bone, 370
  level in bone, 368
Strontium chloride
  soln., for Ca detn. by atomic absorption spectrometry, 201, 202, 204, 207
  urine dilution with for atomic absorption spectroscopy, 43
Stupor, in metabolic alkalosis of children, 391
Sublingual gland, Cl active transport, 254
Succinic dehydrogenase
  activity in kidney
    in Mg deficiency, 324
    in K depletion, 315
Sucrose, renal tubular vacuolization due to, 310
Sugars, active transport, ATPase role in, 240
Sulfalipides, S utilization from, 260
Sulfanilic acid, K detn. with, 23
Sulfatase, sulfate detn. with, 92
Sulfate
  absorption, mechanism, 261
  balance, in anesthesia, 341
  Ca detn. in presence of, 41
  detn., by benzidine method
    calculation, 93
    general aspects, 94
    procedure, 95
    reagents, 94, 95
    standard curve prepn., 95, 96
    urine adaptation, 92, 96
  detn. by chloranilate method, general aspects, 92
  general aspects, 90
  excretion, in lobar pneumonia, 350
  extracellular nature, 91
  interference in Ca detn. by atomic absorption spectrometry, elimination, 204, 205, 207
  by flame photometry, 41, 42
  level in serum
    clinical significance, 263
    in renal insufficiency, 300, 301
    reported values, 4, 5, 92, 96, 159, 260
  loss
    in diabetes, 364
    during tissue catabolism, 297
  metabolism
    in anesthesia, 341
    endocrine control, 262
    regulation, 260-264
    in renal disease, 308
    in vit. A deficiency, 262
  removal for Na detn., 8
  ubiquitousness in animal bodies, 3
Sulfation factor, pituitary function and, 262

Sulfatides, S utilization from, 260
Sulfonamides, intoxication renal parenchymal calcification in, 321
Sulfur
  conjugation products with, 261, 262
  detn. by X-ray spectroscopy, 215, 216, 223
  forms in erythrocytes, 262
  forms and sources, 260, 261
  forms and values for blood vs. blood serum, 262
  level in blood, values by X-ray spectrorometry, 225, 226
  level in bone, 368
  level in serum proteins in multiple myeloma, 216, 217
  loss, in burn trauma, 399
  standard soln. for X-ray spectroscopy, 211, 218
Sulfuric acid
  -ferric sulfate mixture, for serum Na detn., 9
  molybdate reagent prepn. with, 101
  P detn. with, 98
  in K detn. by chloroplatinate method, 32
  -potassium permanganate mixture, Cl detn. with, 75
  soln.
    for Ca detn. by Clark-Collip method, 48, 49
    for ketone body detn., 110
    for lactate detn., prepn., 107
    for Mg detn., prepn., 63
    for P detn., 101
    for total base detn., 163
Sulkowitch test for Ca derangements, 43
Sunderman-Carroll method for Ca and Mg in serum, 199-208
Sunderman conductivity cell
  constant detn., 173
  description, 173
  electrode decoating and replatinization, 172, 173
  figure, 167
  standardization, 173, 174
Sunderman conductivity method for serum total base
  apparatus, 167, 169, 173, 174
  calculations, 174
  clinical aspects, 161
  normal values, 174
  principle, 172
  procedure, 174
  reagents, 172, 173
Sunderman micro-gravimetric method for Na detn., 9, 13-15
Sunderman tetraphenylboron method for K detn., 25
Surgery
  electrolyte changes during, 337, 339
  trauma, anesthesia depth response to, 341
Sweat
  Cl detn. in, mercuric chloranilate method, 79
  Cl level in, normal values, 85
  Cl loss in, 253, 255
Sweating
  Cl and K loss during, 257
  fluid and electrolyte loss due to in infectious diseases, 392
Sympathomimetic amines, gas chromatography, 113
Syringe, tuberculin, for blood collection in pH electrometric detn., 150, 151

T

Tannic acid, burn therapy with, 394
Tartaric acid, N-hydroxynaphthalimide Ca reagent prepn. with, 51
Tartrate buffer
  standard
    composition, 152
    pH values and temp. corrections, 151
Taurocholate
  Na-dependent transport, 233
  S incorporation in, 261
  as S source, 260
Taylor-Miller studies on phosphomolybdic acid complex reduction in acid medium, 97
Teloh conductometric titration of Cl, 76
Teloh method for Mg detn., 57
Temperature, hydrogen ion activity dependence on, 146-148, 151
Testosterone, SO_4 metabolism response to, 262
Tetany
  in aldosteronism, 229
  in hypernatremia of children, 390, 391
  Mg deficiency and, 65, 249
  in metabolic alkalosis of children, 391
Tetraphenylboron method for K
  calculations, 35
  micro-modification, 35
  vs. platinic chloride method, 35
  principle, 33
  procedure, 34, 35
  range of values, 30
  reagents, 33, 34
Tetroxalate buffer, standard, pH values and temp. corrections, 151
Thallium, by X-ray spectroscopy, 218
Thiamine, as S source, 260
Thiazides, diuretic, Cl imbalance due to, 258
Thiers-Hviid apparatus and method for Ca detn., 41
Thiocyanate, Cl titration with in Volhard method, 74
Thiosulfate-starch reaction, in Cl iodometric detn., 75
Thirst, regulation, 273-275
Thymidine

  - Na-dependent transport, 233
  - tritiated, liver regeneration studies with, 360
- Thymol, sulfate detn. with, 91
- Thymus gland, nuclei, Na-dependent non-electrolyte transport, 233
- Thyroid gland
  - Ca and P metabolism control by, 243, 244
  - function, SO_4 metabolism and, 262
  - hyperfunction
    - bone electrolyte metabolism in, 371
    - renal parenchymal calcification in, 321
- Thyrotoxicosis
  - K depletion due to, 312
  - sporadic periodic paralysis in, 383, 384
- Thyrotropin, SO_4 metabolism response to, 262
- Tin, level in bone, 368
- Tisdall method for Ca detn., Clark-Collip modification, 37
- Tissues
  - breakdown
    - in diabetic acidosis, 365
    - hyperkalemia in, 29
  - burned
    - osmotic pressure, 396
    - K replacement by Na in, 398
  - Ca detn. in, by spectrography, 42
  - Cl detn. in, argentimetric method, 82, 83
  - catalbolism, in febrile states, 297
  - permeability, Ca role in, 242
- Titan Yellow, Mg detn. with, 56
- Tongue, myotonia in paramyotonia congenita, 385
- Trauma, osmoregulatory system response to, 275
- Transudates, fatty acids in, gas chromatography, 117
- Triamcinolone, carbohydrate metabolism response to in familial periodic paralysis, 380, 383
- Trinder method for Ca detn., 42, 43, 51-55
- Triphosphopyridine nucleotide diaphorase, activity in kidney in Mg deficiency, 324
- Tripotassium cobalt salt, formation in uncontrolled cobalti-nitrate reaction for K, 24
- Tuberculosis
  - pulmonary, anesthesia in, 342
  - serum total base loss in, 162
  - water and electrolyte abnormalities in, 278
- Tungstic acid, protein precipitant, 23, 119, 120
- Tumors
  - intestinal obstruction due to, 332
  - malignant, renal parenchymal calcification due to, 321
- Tyrosine, Na-dependent transport, 233

U

- Ultrafiltration, serum Ca and Mg detn. by, 66-73
- Ultrafiltration apparatus, for serum Ca and Mg detn., 66, 67
- Uracil, Na-dependent transport, 233
- Uranyl acetate, in SO_4 detn., 91, 92, 94
- Uranyl magnesium sodium acetate, in Na detn., 9
- Uranyl peroxides, in Na detn., 11
- Uranyl zinc sodium acetate
  - precipitate, prepn., 14
  - Na detn. with, 8-11, 13, 14, 16
- Urates, excretion, in salicylate poisoning, 403
- Urea
  - clearance, in K depletion, 318
  - level in serum in uremia, 302
  - osmotic effects in cardiac failure, 288
  - solns.
    - osmolality, 6
    - water metabolism response to, 275
- Uremia
  - in K depletion, 318
  - serum electrolyte patterns in, 299, 300
  - serum electrolyte and non-electrolyte patterns in, 301-305
  - serum total base values in, 161
- Uric acid, level in serum in uremia, 302
- Uridine triphosphate, as glycogen metabolism intermediate in familial periodic paralysis, 379
- Urine
  - aromatic acids in, gas chromatography, 113
  - Ca detn. in
    - by AutoAnalyzer, 40, 41
    - by chelation method, 37, 40
    - by flame photometric method, 41
    - by fluorometric method, 39
    - by N-hydroxynaphthalimide method, 42, 43
    - by nuclear fast red method, 37
  - Ca level in, Sulkowitch test and, 43
  - Ca and Mg detn. in, by atomic absorption spectroscopy, 43
  - Ca and P detn. in, AutoAnalyzer method, 41
  - Cl detn. in
    - argentimetric method, 82, 83
    - colorimetric methods, 76
    - mercuric chloranilate method, 79, 80
    - Volhard titration method, 74, 75
  - Cl diminution in lobar pneumonia, 349-352
  - Cl level in, normal values, 85
  - Cl loss in, 253
  - composition, in lobar pneumonia, 350
  - concentration, in hypernatremia of children, 389
  - concentration and output in diabetes, 362, 363
  - deproteinization for Cl detn., 79
  - dilution, for atomic absorption spectroscopy, 43
  - glucose removal for ketone body detn., 109, 112
  - ketone bodies in
    - detn. by salicylaldehyde-acetone method, 112
    - value range, 112
  - Mg level, 322
  - osmolality

    in acute intermittent porphyria, 277, 278
    regulation, 273-275
  output, in familial periodic paralysis, 376
  oxalic acid level, serum oxalic acid level and, 106
  K detn. in, by cobalti-nitrite methods, 24
  salicylate anlysis in suspected intoxication, 105
  Na detn. in
    colorimetric method, 11
    photometric method, 15-18
  SO_4 detn. in
    benzidine method, 96
    chloranilate method, 92
    general aspects, 90
  SO_4 forms and vols, in, 262, 263

V

Vanatta method for Na detn., 10
Van Schouwenburg method for Ca detn., 37, 38
Van Slyke method for blood gas analysis, 124
Van Slyke-Carson method for Ca detn., 43
Van Slyke-Neill manometric apparatus for blood gas analysis, 124
Van Slyke-Sendroy nomogram for acid-base factors, 133, 134
Van Slyke-Sendroy nomogram for Henderson-Hasselbalch equation soln., 132
Vapor-pressure
  lowering, body fluid osmotic pressure detn. by, 6
  osmometry, serum osmolality detn. by, 181-188
Varley method for Ca detn., 40
Vascular fluid, body per cent composition by weight, 3
Vasodilation, peripheral, in Mg deficiency, 323
Vasopressin, *See* Antidiuretic hormone.
Ventilation (pulmonary) abnormalities
  acid-base disturbances in, 137, 138
  during anesthesia, 342
  acid-base balance and, 270
  alveolar
    CO_2 output and alveolar pCO_2 as related, 267
    efficiency, serum pCO_2 as index of, 135
  body positional effects and, 343, 344
  hyper-
    blood and cerebrospinal fluid pH differentials in, 268, 270, 271
    Cl imbalance due to, 257
    serum electrolyte response to, 341
  respiratory vol. in salicylate intoxication, 402
  role in blood and cerebrospinal fluid pH shifts, 265, 266, 267
Vibrio cholerae, Na transport inhibitor from, 281
Vitamin A, deficiency, SO_4 metabolism in, 262
Vitamin B_6, depletion, liver regeneration in, 360
Vitamin B_{12}, depletion, liver regeneration in, 360
Vitamin C, Ca and P metabolism response to, 246
Vitamin D
  Ca and P metabolism response to, 243, 246
  intoxication, renal parenchymal calcification in, 321
Violuric acid, in Na detn., 9, 12
Volhard method for Cl in serum and urine, 74-76
Volvulus, intestinal obstruction due to, 332

W

Watson-Rogers method for Ca detn., 40
Water
  balance
    abnormalities, 276-279
    in anesthesia, 337-348
    in heart failure, 288-292
    in hypernatremia of children, 389
  CO_2-free, prepn., 125
  exchange between blood and interstices, 7
  imbalance
    in cirrhosis, 353, 354
    in liver disease, 353-361
  intake, in hypernatremia of children, 389
  intoxication
    in heart failure therapy, 291
    in parenteral therapy for hypernatremia in children, 391
    prevention, in acute intermittent porphyria, 278
  level in edema fluid, in burn trauma, 397
  level in kidney, in hypokalemia, 316
  metabolism, abnormalities
    in acute intermittent porphyria, 277, 278
    hormonal, 276-279
    in tuberculosis, 278
    hypothalamic-neurohypophyseal regulation, 273-279
    in intestinal obstruction, 332-336
    notation units, 4
    pitressin effects on, 277
  potassium sodium cobalti-nitrite precipitate solubility in, 21, 25
  uptake by muscle in familial periodic paralysis, 376, 377, 383
Waxes, medicated, burn therapy with, 394
Weakness, in aldosteronism, 229
Webb-Wildy method for Ca detn., 42
West-Coll method for Cl detn., 76
Whipple's disease, concommitant with parathyroid hyperplasia vacuolar nephropathy in, 312
White method for sulfate, 90
Wieme-Van Raepenbusch AutoAnalyzer method for Da, 40

Wilkinson method for Mg detn., 57
Williams-Moser method for Ca detn., adaptation to AutoAnalyzer, 40
Willis method for Ca and Mg detn., 43
Willis-Sunderman nomogram for ultrafiltrable Mg, 70-72
Wilson-Ball method for Cl detn., 76
Wilson's disease, renal parenchymal calcification in, 321

X

X-rays, emission spectrometry, serum electrolyte detn. by, 209-227

Y

Yalman method for Ca detn., 40
Yanagisawa method for Ca detn., 39
Yarbro-Golby method for serum Ca detn., 38
Yasuo, N-hydroxynaphthimide reagent for Ca detn., 51
Yoshimatsu method for sulfate, 90, 91

Z

Zac method for Mg detn., 57
Zinc
  detn. by X-ray spectroscopy, 214
  electroplating for X-ray spectroscopy, 214
  level in blood, clinical interpretation, 226
  level in blood and blood cells, values by X-ray spectroscopy, 225, 226
  standard soln. for X-ray spectroscopy, 211, 219
Zinc sulfate
  -barium hydroxide combination
    body fluid deproteinization with for ketone body detn., 109
    for lactate detn., 107
Zollinger-Ellison syndrome, K depletion in, 312

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